The Molecular Mechanism of Chronic δ-Opioid-Mediated Adenylyl Cyclase Superactivation in Chinese Hamster Ovary Cells Stably Expressing the δ-Opioid Receptor: A Cellular Model for Tolerance and Withdrawal

By

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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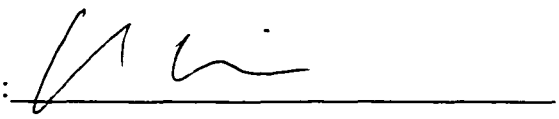
SIGNED: 

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DEDICATION

Dr. Henry Yamamura has been one of my mentors for presenting my results, interacting with other scientists, and teaching. His outstanding professionalism is evidenced by his continually dynamic and successful career in research, highly sought-after laboratory, and Regents' professorship. Dr. Yamamura leads by example and, as I learned soon after I began work in his laboratory, he understands that this method of teaching is both constructive and effective.

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ABSTRACT

We are studying the pharmacology of the human δ-opioid receptor stably expressed in Chinese hamster ovary cells (hDOR/CHO). The δ-opioid receptor mediates analgesia, without the negative side effects noted by μ- and κ-opioid agonists. However, tolerance to δ-opioid agonists still occurs. One mechanism of drug tolerance in CHO cells involves a compensatory response by adenylyl cyclase VI. This response, termed adenylyl cyclase (AC) superactivation, arises from the observation that acute hDOR activation leads to inhibition of AC, whereas chronic activation of the receptor (greater than 4 hours) leads to a compensatory increase in AC activity, effectively negating the acute inhibition normally seen in the presence of the δ-opioid agonist. The increased AC activity also causes an overshoot of cAMP formation upon the removal of the agonistic inhibitory influence. The loss of receptor-mediated AC inhibition after chronic agonist treatment is thought to contribute to *in vivo* drug tolerance, and the resulting cAMP overshoot may contribute to opioid withdrawal. In studying this phenomenon, we have demonstrated a requirement for G-protein βγ subunits ($G_{\beta\gamma}$) by expressing scavengers of $G_{\beta\gamma}$, such as α-transducin and phosducin. Additionally, we have shown AC VI phosphorylation by chronic agonist treatment, which may cause AC superactivation, and that this phosphorylation is sensitive to calmodulin antagonists and inhibitors of the atypical protein kinase C enzymes. We have also recently postulated the involvement of Raf-1 kinase. Inhibitors of Raf-1, as well as pathways that lead to activation of this enzyme, significantly attenuate the cAMP overshoot, suggesting that hDOR-activated Raf-1 can phosphorylate and superactivate AC VI in CHO cells.

CHAPTER 1: INTRODUCTION

People taking medications often experience tolerance. When taken regularly, such drugs lose their effectiveness, thereby requiring additional dosing to offset this loss of activity. The phenomenon is collectively referred to as tolerance. Tolerance to drug therapy is a ubiquitous phenomenon, which can range from being a nuisance to providing pharmacological obstacles to effective long-term therapy.

Tolerance to a drug like caffeine manifests itself in the seasoned coffee drinker as the requirement for more caffeine to achieve the usual desired stimulant response. When the coffee drinker has his usual pot replaced with a decaffeinated variety, he will likely experience a withdrawal, with a splitting headache and fatigue as the major symptoms. These withdrawal symptoms appear to be responses that are directly opposed to the actions of the drug itself. In a state of chronic drug administration, the tolerant individual seems to be able to adapt to unrelenting exposure and reset those affected systems to account for their constant perturbation. By withdrawing the drug, these modified systems find themselves resisting a perturbation that no longer exists, resulting in the expression of symptoms that are opposed to the acute drug action. This *in vivo* observation is fundamental to the validation of *in vitro* studies of tolerance and withdrawal, as will be delineated below.

As I am sure the reader is aware, drugs act at a multitude of targets, each drug falling into a certain class defined by its target. Of all the possibilities, G-protein-coupled receptors comprise the targets of over thirty percent of all clinically marketed drugs, a number that is sure to increase with the recent cloning of the human genome and

subsequent unmasking of the fact that this already impressive thirty percent comprises fewer than ten percent of all the potential G-protein-coupled receptors (Wise, et al., 2002). While it is obviously important to hunt for these novel receptor targets, we must also characterize the physiology of known receptors, as there will undoubtedly be significant parallels between the current and yet undiscovered systems. Not surprisingly, much is already known about the G-protein-coupled receptor superfamily. They are defined by their ability to signal through guanine nucleotide binding proteins (G-proteins), and have seven helical transmembrane regions. They all participate in an enzymatic cycle that begins with the activation of the receptor by an agonist (such as a hormone, neurotransmitter, photon, or drug), and results in the activation or inhibition of various effectors and the downstream modulation of second messenger formation.

Upon binding of a neurotransmitter, the receptor-G-protein complex is activated (**Figure 1**). This allows for dissociation of guanosine diphosphate (GDP) from the α subunit of the G-protein (G_α) and subsequent association of GTP. The $G_{\beta\gamma}$ subunits, tightly associated with the GDP-bound G_α, are now released from GTP-bound G_α. In this way, both activated G_α and liberated $G_{\beta\gamma}$ subunits are free to interact with effectors. Eventually, the inherent GTPase activity of the G_α subunit hydrolyzes its bound GTP back into GDP, allowing for reassociation with $G_{\beta\gamma}$ and a resetting of the system for another round. The cycle continues unabated until the agonist dissociates from its receptor, which can last from milliseconds to minutes.

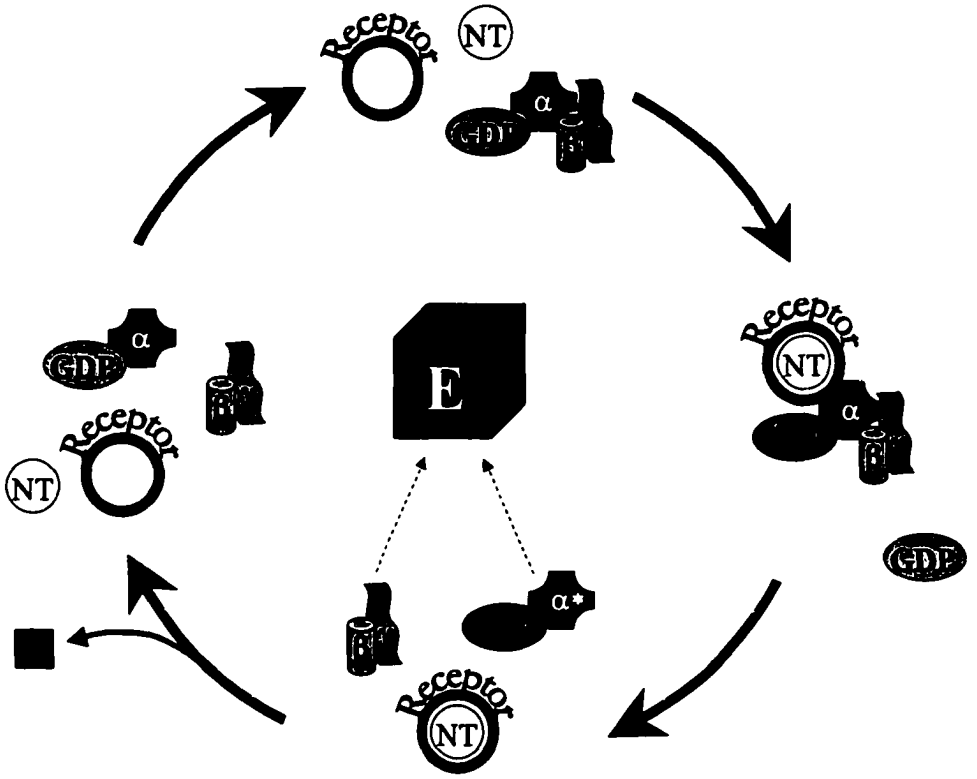

Figure 1: The G-Protein Cycle

When activated, a guanine nucleotide-binding protein (G-protein)-coupled receptor catalyzes a cycle of events that results in the activation or inhibition of various effectors and downstream modulation of second messenger formation. Upon binding of a drug or endogenous neurotransmitter (NT), the receptor-G-protein complex is activated. This allows for dissociation of guanosine diphosphate (GDP) from the a subunit of the G-protein (G_α) and subsequent association of GTP. The $G_{\beta\gamma}$ subunits, normally tightly bound to GDP-bound G_α, are released from GTP-bound G_α. In this way, both activated G_α and liberated $G_{\beta\gamma}$ subunits are free to interact with effectors. Eventually, the GTPase activity of G_α hydrolyzes its bound GTP into GDP, allowing for reassociation with $G_{\beta\gamma}$ and a resetting of the system for another round. The cycle can continue unabated until the neurotransmitter dissociates from its receptor.

Whereas tolerance has been demonstrated for a vast array of drugs, drugs that act at G-protein-coupled receptors, such as morphine and related analgesics, dopamine (for Parkinson's disease), or clonidine (for hypertension), have been intense foci of research due to the prevalence and importance of this type of receptor as a drug target. The above receptors in particular couple to inhibitory G-proteins ($G_{i/o}$), named as such for their ability to *inhibit* adenylyl cyclase and reduce intracellular cyclic 3',5'-adenosine monophosphate (cAMP), a ubiquitous second messenger. Other G-protein-coupled receptors can activate G_s (adenylyl cyclase stimulatory) or G_q (phospholipase C stimulatory) proteins. Because the focus of our research is opioid systems, which involve only $G_{i/o}$-coupled receptors, the remainder of the dissertation will focus primarily on such $G_{i/o}$–coupled systems. These opioid receptors are the receptors activated exogenously by opiates such as morphine and heroin.

Morphine and related opioids are some of the most clinically important and ubiquitously abused substances in the world (Marquet, et al., 1998; Poulin, et al., 1998). Early opioid research led to the discovery of a receptor where morphine acts (Pert and Snyder, 1973; Simon, 1973; Terenius, 1973) and, eventually, to endogenous opioid ligands (Hughes, et al., 1975; Guillemin, 1976; Goldstein, et al., 1979; Zadina, et al., 1997). While the opiates are vital for clinical pain control, they have the regrettable side effects of respiratory depression, constipation, and addiction. The development of novel drugs with the analgesic appeal of morphine, but with limited side effects and abuse liability has been a holy grail of pain research. A fortuitous break came in the form of various opioid receptor types. This multiplicity had been hypothesized and partially

characterized using novel agonists and antagonists that were selective for one type or another. Such studies resulted in the discrimination of three opioid receptors, named μ, κ, and δ. Eventually, advances in our understanding of the molecular biology of opioid systems led to the cloning of each of these opioid receptors types (Kieffer, et al., 1992; Chen, et al., 1993; Minami, et al., 1993). Although the respective roles of the μ-, κ-, and δ-opioid receptors are still evolving, it appears that the δ-opioid receptor can mediate analgesia as effectively as morphine, but may not produce morphine's respiratory depression, constipation, prolactin secretion, or abuse potential (Rapaka and Porreca, 1991). Nevertheless, analgesic tolerance still develops to δ-opioid agonists (Schulz, et al., 1981), ultimately limiting their pharmacological utility. Due to its potential as a potent and effective analgesic mediator, we have chosen to study tolerance and withdrawal in the δ-opioid system.

The human δ-opioid receptor was cloned in 1994 in our laboratory by Richard Knapp (Knapp, et al., 1994), and subsequently stably transfected into Chinese hamster ovary (CHO) cells (Malatynska, et al., 1995). We investigate δ-opioid pharmacology, such as tolerance and withdrawal, in this heterologous expression system by measuring downstream effector activity after exposure to acute or chronic periods with a δ-opioid agonist. As will be discussed in detail, we have shown that acute agonist treatment results in a decrease in cAMP formation, while chronic treatment followed by removal drastically increases cAMP formation. This phenomenon is commonly referred to as the cAMP overshoot or, where applicable, adenylyl cyclase superactivation.

The molecular events that transpire to produce drug tolerance in humans are complex and likely involve a multitude of tissues and cellular modifications (Nestler, 1993; Mao, et al., 1995; Liaw, et al., 1996). Therefore, simpler *in vitro* cellular models with fewer variables are attractive for tolerance and withdrawal research. This has resulted in the study of isolated intact tissues, primary cell cultures of various tissues, and untransfected cell lines that all appear to naturally exhibit drug tolerance, as well as cell lines transfected with particular investigative proteins necessary for demonstrating tolerance in such expression systems. It is hoped that this reductionist approach will eventually lead to the synthesis and formulation of a generalized theory for drug tolerance and withdrawal, or at least to identification of the minimal necessary elements required for induction of tolerance in any given tissue. In any case, the sequence of events leading to drug tolerance and withdrawal must begin with receptor activation.

Many cellular responses occur in response to chronic G-protein-coupled receptor activation. The activated receptors are often phosphorylated by G-protein-coupled receptor kinases (GRKs), which encourage the association of the β-arrestins (Benovic, et al., 1987) with the receptor. These proteins serve to stearically uncouple the receptor from its cognate G-proteins. Furthermore, agonist-bound, phosphorylated receptors can endocytose (internalize) and be sent to one of two pathways: The majority of internalized receptors are dephosphorylated and recycled back to the membrane surface, whereas a small fraction is targeted for degradation. If allowed to run for many internalization cycles, as in the case of chronic agonist exposure, a significant number of receptors can be degraded, leading to receptor down-regulation (for review, see (Tsao and von Zastrow,

2000)). While these two processes occur at the receptor level, other forms of signal attenuation occur at downstream effectors. For example, G-protein-coupled receptors that activate inhibitory G-proteins acutely inhibit adenylyl cyclase, while these same systems can typically compensate for this chronic inhibition by increasing the activity or expression of adenylyl cyclase. This represents a form of receptor-effector negative feedback (Gintzler and Chakrabarti, 2000). Collectively, desensitization, down-regulation, and effector negative feedback all serve to diminish G-protein-coupled receptor signaling, thereby maintaining intracellular homeostasis in light of chronic receptor activation. While *in vivo* tolerance to drugs like morphine may involve more than just these three molecular events (Hsu and Wong, 2000), it seems likely that these cellular changes sum to significantly contribute to tolerance (Alvarez, et al., 2001).

Although people have been cognizant of drug tolerance for a very long time (Way, 1979), the first subcellular mechanistic exploration of this phenomenon occurred in 1975 in the laboratory of Marshall Nirenberg. They created a neuroblastoma-glioma hybrid cell line, which they called NG108-15. In these cells, they demonstrated both acute and chronic modulation of adenylyl cyclase by measuring intracellular cAMP concentrations (Sharma, et al., 1975a; Sharma, et al., 1975b). In their studies, NG108-15 cells, which we now know endogenously express mouse δ-opioid receptors, exhibited a typical inhibition of cAMP formation in response to acute treatment with morphine, whereas chronic morphine treatment followed by removal of the agonist (i.e. withdrawal) resulted in a significantly increased cAMP accumulation (both basal and PGE_1-stimulated), referred to as a cAMP overshoot (**Figure 2**). This is notable because the

cAMP overshoot has since been shown to be an important mediator of *in vivo* opioid withdrawal (Tokuyama, et al., 1995; Tokuyama, et al., 1998; Noda, et al., 2001). Furthermore, the cAMP overshoot has been demonstrated in many different opioid receptor-expressing tissues treated chronically with opioid agonists, such as *in vivo* brain (Collier and Francis, 1975; Collier, et al., 1975; Maldonado, et al., 1996), specific

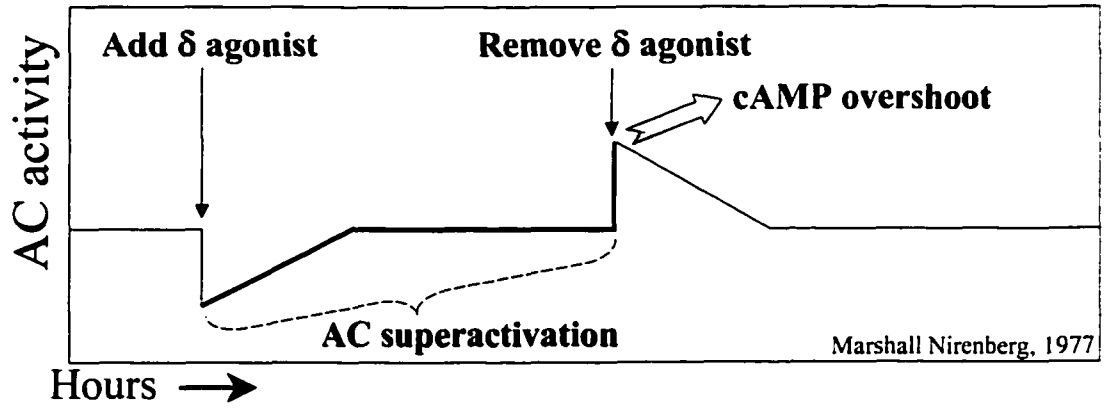

Figure 2: Schematic of Adenylyl Cyclase Superactivation

Adenylyl cyclase superactivation as, originally described by Sharma et al. (1977), occurs by the cell augmenting the activity its adenylyl cyclase during a long-term exposure to an inhibitory agonistic influence. The increase exactly matches the inhibition to cancel out the inhibitory effect of the agonist on adenylyl cyclase. The formation of adenylyl cyclase superactivation begins within minutes of the exposure, but requires hours to reach completion. This phase has been compared to the tolerance induced by such agonist drugs in the whole animal. When the agonist is removed by several buffer washes, the increased adenylyl cyclase activity results in an overshoot of cAMP formation. Because cAMP is an important factor in the opioid withdrawal syndrome in the whole animal, this phase of adenylyl cyclase superactivation has been compared with withdrawal. In these ways, adenylyl cyclase superactivation has been considered a viable cellular model for opioid tolerance and withdrawal.

isolated brain regions (Maldonado, 1997), intestine (Chakrabarti, et al., 1998a), *in vitro* cell lines (Sharma, et al., 1975a), and $G_{i/o}$-protein-coupled receptor heterologous expression systems (Nestler, 1993; Malatynska, et al., 1996; Maldonado, et al., 1996; Blendy and Maldonado, 1998; Rubenzik, et al., 2001). Because the cAMP overshoot is generally mediated by an increase in adenylyl cyclase activity, it is often referred to as adenylyl cyclase superactivation.

Chronic opioid agonist-mediated adenylyl cyclase superactivation and the resultant cAMP overshoot have long been considered *in-vitro* models for studying opioid tolerance and withdrawal (Sharma, et al., 1975b; Sharma, et al., 1977; Nestler, 1993; Malatynska, et al., 1996; Ammer and Schulz, 1998). Although Sharma et al.'s (1975a) finding took place more than a quarter century ago, the molecular mechanism, from receptor activation to increased cAMP formation, of what is now termed adenylyl cyclase superactivation is still not entirely understood. What has been shown is the involvement of certain adenylyl cyclase isoforms, G-protein α and $\beta\gamma$ subunits, and kinases (Avidor-Reiss, et al., 1995; Ammer and Schulz, 1998; Chakrabarti, et al., 1998a; Chakrabarti, et al., 1998b). Yet, the mechanism of adenylyl cyclase superactivation remains rather controversial and there appear to be a number of components that are required to demonstrate it:

Ammer and Schulz (1998) have established the requirement for activated $G_{\alpha s}$ to show a cAMP overshoot in opioid-withdrawn NG108-15 cells (Ammer and Schulz, 1998). Chakrabarti et al. (1998a) have shown that in guinea pig ileum longitudinal muscle myenteric plexus preparations, pretreatment *in vivo* with chronic morphine results

in a type of cAMP overshoot wherein a reversal from receptor-mediated inhibition to stimulation of adenylyl cyclase is observed (Chakrabarti, et al., 1998a). Additionally, they demonstrated that activation of PKC is required for ACII phosphorylation by the same *in vivo* pretreatment (Chakrabarti, et al., 1998b) and that ACII was upregulated (Chakrabarti, et al., 1998a), which is an adenylyl cyclase that has been shown to be conditionally stimulated by $G_{\beta\gamma}$ and $G_{\alpha s}$ (Sunahara, et al., 1996). It is likely that, in this system, all of these events contribute to the cAMP overshoot caused by chronic morphine. In contrast, a CHO cell line stably transfected with the rat μ-opioid receptor exhibits adenylyl cyclase superactivation that is unaffected by cycloheximide pretreatment, indicating that no up-regulation of adenylyl cyclase or $G_{\alpha s}$ occurs in this system (Avidor-Reiss, et al., 1995). In sum, these results indicate that, while the cAMP overshoot phenomenon first demonstrated by Sharma et al. (1975a) is common, the series of events leading up to this response is not well understood. Some aspects of the cAMP overshoot, however, appear to be consistent, such as signaling through $G_{\beta\gamma}$.

The aim of this dissertation research has been to determine, and characterize where possible, key steps in the molecular process that begins with δ-opioid receptor activation and ends with superactivation of adenylyl cyclase in Chinese hamster ovary cells. Our initial hypothesis was that the hDOR/CHO cells represent a useful model system for studying adenylyl cyclase superactivation. We began by further characterizing the Chinese hamster ovary cells stably expressing the human δ-opioid receptor (hDOR/CHO cells), originally partially characterized by Ewa Malatynska (Malatynska, et al., 1996). From there, our goal was to examine the possible signal transduction pathways

utilized by the δ-opioid receptor in these cells, assuming that one or more of these pathways superactivate the adenylyl cyclase. This led to our central hypothesis that $G_{\beta\gamma}$ subunits, derived from pertussis toxin-sensitive $G_{\alpha i/o}$-proteins, are required for adenylyl cyclase superactivation. Understanding what is known about δ-opioid receptor signal transduction is therefore essential to understand the basis for this hypothesis (**Figure 3**).

The $G_{\alpha i/o}$-protein α-subunits that are activated by δ-opioid receptor stimulation are responsible for the acute inhibition of adenylyl cyclase. Only one other putative role has been demonstrated for such $G_{\alpha i/o}$ subunits, which is to activate Src, a protein tyrosine kinase that can lead to mitogen-activated protein kinase (MAPK) activation (Ma, et al., 2000). The slew of additional responses mediated by $G_{i/o}$-protein-coupled receptors is by the liberated $G_{\beta\gamma}$ subunits. Such responses have been shown to include Src activation, phosphatidylinositol-3-kinase (PI-3K), and phospholipase Cβ (PLCβ) activation. Activated PI-3K converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphtitylinositol-3,4,5-trisphosphate (PIP_3), which can activate PLCγ and certain isoforms of PKC (Wymann and Pirola, 1998).

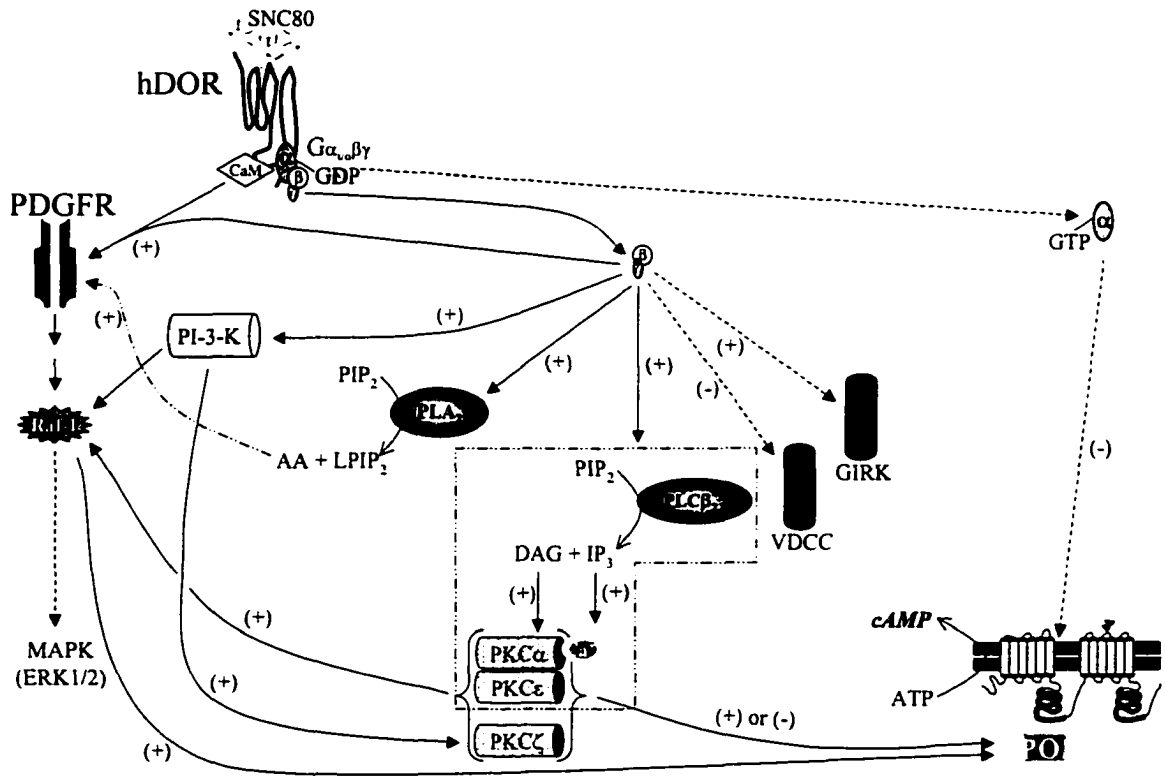

Figure 3: δ-Opioid Receptor Signal Transduction Pathways

These are many of the pathways that have been shown to be mediated by the δ-opioid receptor. The $G_{\beta\gamma}$-mediated modulation of voltage-dependent calcium channel (VDCC) and G-protein inward rectifying potassium channel (GIRK) only occur in excitable tissues. The remaining pathways involve δ-opioid-mediated modulation of ubiquitous signaling molecules.

Abbreviations: hDOR (human δ-opioid receptor), CaM (calmodulin), GDP (guanosine diphosphate), GTP (guanosine triphosphate), PDGFR (platelet-derived growth factor receptor), PI-3-K (phosphaitylinositol-3-kinase), PLA_2 (phospholipase A_2), PIP_2 (phosphatidylinositol bisphosphate), AA (arachidonic acid), $LPIP_2$ (lysophosphatiditylinositol bisphosphate), PLCβ (phospholipase Cβ), DAG (diacylglycerol), MAPK (mitogen-activated protein kinase), PKC (protein kinase C), ATP (adenosine triphosphate).

The family of phospholipase C enzymes hydrolyzes phosphatidylinositol diphosphate (PIP_2) to form diacylglycerol (DAG) and inositol trisphosphate (IP_3). Their enzymatic activity requires a calcium ion as a cofactor. There are 3 main classes of phospholipase C (PLC) families: PLCδ, PLCγ, and PLCβ, which differ in their regulatory mechanisms and means of activation. Four PLCδ isoenzymes ($PLC\delta_{1-4}$) have been cloned. PLCδ enzymes can be directly stimulated by free calcium. In contrast, PLCγ is regulated mainly by receptor and non-receptor tyrosine kinases and PIP_3. Two PLCγ isoenzymes are known. When a receptor tyrosine kinase is autophosphorylated, PLCγ can interact with the phosphotyrosines and be phosphorylated and activated. The PLC enzymes that are coupled to G protein activation are of the β type. Thus far, four PLCβ isoenzymes have been extensively characterized ($PLC\beta_{1-4}$). They differ mainly in their ability to be stimulated by $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits: $PLC\beta_2$ and $PLC\beta_3$ are more sensitive to $G_{\beta\gamma}$ subunits than the $PLC\beta_1$ and $PLC\beta_4$ enzymes. Dickenson and Hill performed Western blots in CHO cells for $PLC\beta_{1-3}$, and found that, of these isoforms, CHO cells contain only $PLC\beta_3$ (Dickenson and Hill, 1998). One product of PLC activation, IP_3, increases intracellular calcium by acting at ionotropic IP_3 receptors in the endoplasmic reticulum.

The other PLC product, DAG, can activate two of the four families of protein kinase C (PKC): the calcium-dependent conventional PKCs (PKCα, βI, βII, and γ) and the calcium-independent novel PKCs (PKCδ, ε, η, and θ). The third PKC form, known as the atypical PKCs (PKCι, λ, and ζ) is not activated by DAG or calcium, but by PIP_3 (the

product of PI-3K activation), whereas little in known about the fourth form, PKCμ (for review, see (Mellor and Parker, 1998)). It seems that conventional, novel, and atypical PKC isoforms can be activated by opioid receptors (Kramer and Simon, 1999). Furthermore, members from each of these three isoforms are endogenously expressed in Chinese hamster ovary cells (Megson, et al., 2001). PKC phosphorylation of adenylyl cyclase can inhibit or enhance enzymatic activity, depending on the adenylyl cyclase isoform in question.

Multiple isoforms of adenylyl cyclase also exist. This is important as each of the nine membrane-bound adenylyl cyclase isoforms (ACI – ACIX) are regulated very differently. While all the isoforms can be stimulated by $G_{s\alpha}$ and forskolin and inhibited by P-site analogs (ATP antagonists), adenylyl cyclases are classified into four groups, depending on their divergent regulatory mechanisms. Group 1, containing ACI, III, and VIII, can be activated by Ca^{++}/calmodulin and inhibited by $G_{\alpha i}$, $G_{\beta\gamma}$, and Ca^{++}/calmodulin-dependent protein kinase IV (CaMK IV) phosphorylation. Group 2, containing ACII, IV, and VII, are activated by $G_{\beta\gamma}$ and PKC. Group 3, containing ACV and VI, can be activated or inhibited by PKC (possibly depending on the PKC isoform), but are inhibited by $G_{\alpha i}$, $G_{\beta\gamma}$, μM Ca^{++}, and PKA. The putative structure of mammalian adenylyl cyclase (**Figure 4**) is that of two symmetrical halves, each with six highly conserved (among AC isoforms) transmembrane spans. The first large cytoplasmic loop contains the C1a and C1b regions, whereas the second large cytoplasmic domain contains the C2a and C2b regions. C1a and C2a are homologous to each other and presumably, each contain half of the catalytic site.

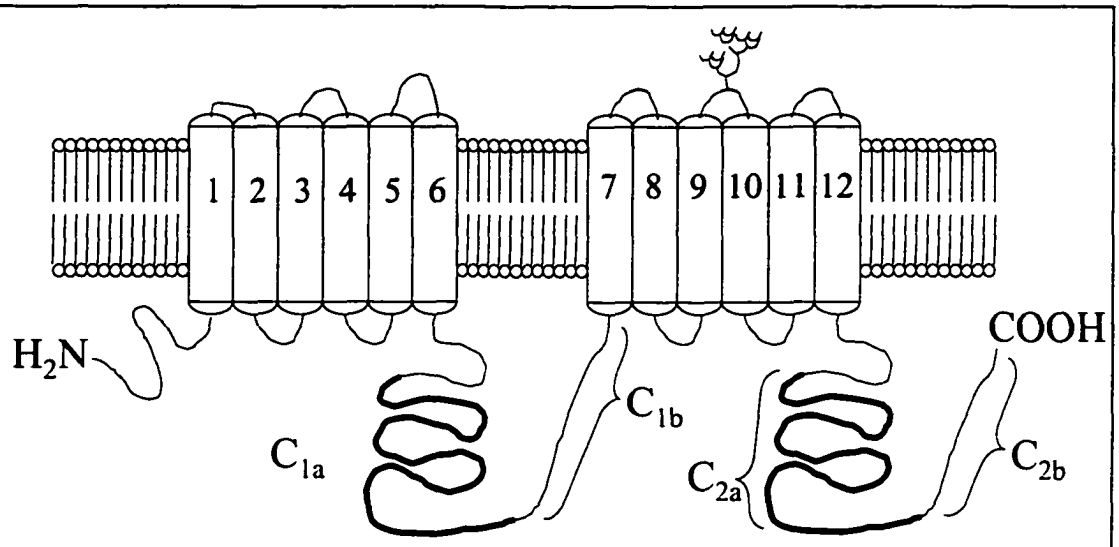

Figure 4: Structure of Adenylyl Cyclase

Adenylyl cyclase consists of two highly homologous regions, each containing six transmembrane domains. The intracellular domain between these regions contains the C1a and C1b domains, whereas the carboxyl tail contains the C2a and C2b domains. When the C1a and C2a domains are pulled into proximity by forskolin or $G_{\alpha s}$, the enzyme is activated.

When proteins, such as $G_{\alpha s}$, or drugs, such as forskolin, interact with the C1b and/or C2b regions, the catalytic C1a and C2a regions are pulled into proximity of one another, forming the active catalytic site. Adenylyl cyclase phosphorylation constitutes a ubiquitous regulatory mechanism. Phosphorylation by PKC generally results in activation (although this is controversial), whereas phosphorylation by PKA or CaMK results in inhibition of the enzyme. These pathways presumably serve as negative feedback mechanisms for the regulation of adenylyl cyclase. All adenylyl cyclase isoforms can be found in the brain, but not all adenylyl cyclases are capable of superactivation. Of the

nine characterized adenylyl cyclases, only ACI, V, VI, and VIII appear to exhibit superactivation (Avidor-Reiss, et al., 1997; Nevo, et al., 1998).

A relatively novel signal transduction pathway is currently being elucidated involving G-protein-coupled receptor-mediated activation of mitogen-activated protein kinase (MAPK) (Gutstein, et al., 1997; Kribben, et al., 1997; Hedin, et al., 1999). This discovery is complicated by the demonstration of multiple routes for arriving at MAPK phosphorylation. The first is through a process of transactivation (Berhow, et al., 1996; Schulz and Hollt, 1998; Tso and Wong, 2001; Shah and Catt, 2002). In this paradigm, a tyrosine kinase growth factor receptor (e.g. epidermal growth factor receptor, EGFR, or platelet-derived growth factor receptor, PDGFR) can be activated by the non-receptor tyrosine kinase Src, originating from either direct $G_{\alpha i/o}$- or $G_{\beta\gamma}$/PI-3K-stimulation. Another, as yet uncharacterized pathway involves the $G_{\beta\gamma}$-, calcium/calmodulin- and/or PKC-dependent activation of membrane metalloproteases, which cleave EGF or PDGF precursors to EGF or PDGF, allowing transactivation of these receptors by direct interaction with their respective growth factor ligands (Belcheva, et al., 2001). A third pathway requires the G-protein-coupled receptor binding protein, β-arrestin. In this scheme, an activated receptor, phosphorylated by a G-protein-coupled receptor kinase (GRK), binds intracellular β-arrestin. The associated β-arrestin can then act as a scaffold for Raf-1 kinase (a MAPKKK), MEK (a MAPKK), and ERK2 (a MAPK), and putatively additional members of the ERK1/2 cascade (Luttrell, et al., 2001; Tohgo, et al., 2002). To further confuse matters, a tyrosine receptor transactivation- and Ras-*independent*

pathway, involving direct PKCα- and PKCε-mediated phosphorylation and activation of Raf-1, has been noted to lead to ERK activation (Cai, et al., 1997).

Our central hypothesis, that adenylyl cyclase superactivation is mediated by $G_{\beta\gamma}$ subunits, results from the observation that the bulk of the receptor responses require $G_{\beta\gamma}$. After characterizing the hDOR/CHO system, we tested this hypothesis by expressing a $G_{\beta\gamma}$ scavenger, thereby limiting the ability of the δ-opioid receptor to signal through $G_{\beta\gamma}$. Once the requirement for $G_{\beta\gamma}$ was demonstrated, we set out to determine which $G_{\beta\gamma}$-mediated pathway(s) might be involved in adenylyl cyclase superactivation by examining various modulators of adenylyl cyclase as well as the adenylyl cyclase itself.

As shall be discussed in this dissertation, we have demonstrated the requirement for G-protein βγ subunits derived from pertussis toxin-sensitive G-protein α subunits, determined precisely which α subunit and adenylyl cyclase isoforms are expressed in CHO cells, revealed increased adenylyl cyclase phosphorylation with a similar time-course and potency to superactivation, and established the involvement of calmodulin. From these data, we have developed a generalized model for how adenylyl cyclase superactivation may be occurring in CHO cells stably expressing the human δ-opioid receptor.

CHAPTER 2: CHARACTERIZATION OF THE HUMAN δ-OPIOID RECEPTOR IN CHINESE HAMSTER OVARY CELLS

INTRODUCTION

In 1994, Knapp et al. (1994) cloned the human δ-opioid receptor from a human brain library (Knapp, et al., 1994). Stable transfection of Chinese hamster ovary (CHO) cells with the human δ-opioid receptor thereafter resulted in the establishment of a cell line stably expressing a high δ-opioid receptor density (2-209-2CHO or hDOR/CHO cells) (Malatynska, et al., 1995). This clone was originally characterized by [^{3}H]-naltrindole binding, SNC80 and DPDPE inhibition of forskolin-stimulated cAMP formation, and the effects of long-term (24 hr) SNC80 studies in down-regulation of the receptor and the cAMP overshoot (Malatynska, et al., 1995; Malatynska, et al., 1996). In the process of extensive further characterization, I have reproduced and verified some of this work.

The density of the expressed human δ-opioid receptor in CHO cells can change over the years of cell culture and continual selection. I performed saturation binding assays using [^{3}H]-naltrindole, a selective high affinity δ-opioid antagonist to determine the current δ-opioid receptor expression. Because the original B_{max} value was very high (about 137,000 ± 21,600 receptors per cell (Malatynska, et al., 1995)) when determined in 1995, even substantial increases in expression would probably not affect the function of the receptor due to vast receptor reserve. When the hDOR/CHO clone was created, SNC80 was able to inhibit forskolin-stimulated cAMP formation nearly 100%. We

expected this observation to remain consistent in spite of some indication by others in the laboratory that human δ-opioid receptor expression had decreased some over the years.

We measured the abilities of multiple δ-opioid ligands to affect forskolin-stimulated cAMP formation. We tested SNC80, deltorphin II, (-)TAN67, and the inverse agonist, ICI 174,864, to inhibit or activate adenylyl cyclase. We also examined the pertussis toxin sensitivity of SNC80-mediated inhibition of adenylyl cyclase. These experiments were important to investigate the capabilities of the hDOR/CHO cell line. We expected our clone to represent a realistic and predictable expression system consistent with the basic characteristics of related cell lines.

Further characterization of hDOR/CHO cells included coupling of the human δ-opioid receptor to inositol phosphate formation via PLCβ activation. We examined SNC80-mediated IP_1 formation in the presence of lithium (to inhibit degradation of the formed IP_1). In many opioid receptor expression systems, this response has been shown to be mediated by $G_{\beta\gamma}$ subunits. We also inspected the pertussis toxin sensitivity of this response to determine if the $G_{\beta\gamma}$ subunits responsible for PLCβ activation were derived from $G_{\alpha i/o}$ G-proteins.

As stated, early characterization of the hDOR/CHO cell line was comprised of two long-term agonist pretreatment studies. One of these resulted in the discovery of adenylyl cyclase superactivation in this cell line. The other demonstrated agonist-mediated desensitization of adenylyl cyclase inhibition by SNC80. In this paradigm, pretreatment of these cells with 100 nM SNC80 for 24 hours resulted in the decreased ability of SNC80 to mediate inhibition of forskolin-stimulated cAMP formation as a

percent of control. This being quite interesting, we investigated the fate of the PLCβ activation after long-term agonist pretreatment. We expected to observe an attenuation of IP_1 formation in this pretreatment protocol.

Finally, we thought it important to know which of the five possible $G_{\alpha i/o}$ subunits ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o1}$, $G_{\alpha o2}$, $G_{\alpha t}$) were expressed in the CHO cells. We figured that such knowledge would facilitate our understanding of the molecules involved in the pathways activated by the human δ-opioid receptor. We accomplished this by isolating mRNA from CHO cells and amplifying the pertussis toxin sensitive G-protein transcripts using primers directed towards conserved regions in these genes.

General Methods

Forskolin-Stimulated cAMP Formation (modified from (Gilman, 1970))

The growth medium was aspirated and replaced with serum-free Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Carlsbad, CA). In experiments involving opioid agonist pretreatment, either IMDM or 100 nM SNC80 (Tocris, Ballwin, MO) in IMDM was added to cells for 4 hours. Following this chronic treatment, the cells were washed three times with fresh IMDM, fifteen minutes per wash. The IMDM was then aspirated and replaced with 5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical, St. Louis, MO) in IMDM. Adenylyl cyclase was stimulated with water-soluble forskolin (7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl, diHCl) (Calbiochem, San Diego, CA). Doses of SNC80 with 100 μM forskolin (agonist dose-response curve) or doses of forskolin alone (forskolin dose-response curve) were added to the wells, which

were then placed in a humidified incubator at 37° C (5% CO_2) for 20 minutes. The forskolin was then aspirated and replaced with ice-cold 150 µl Tris/EDTA buffer (50 mM Tris HCl , 4 mM EDTA, pH=7.5) to terminate the reaction. Each well was scraped to dislodge the cells and the contents of each well were transferred to microfuge tubes (Beckman Coulter, Fullerton, CA) and boiled for 10 minutes to lyse the cells. Lysate was centrifuged and 50 µl of supernatant was incubated with 50 µl [^{3}H]-cAMP (Perkin Elmer Life Sciences, Boston, MA) (4 nM final concentration) and 100 µl PKA (Sigma Chemical, St. Louis, MO) (30 µg/ml final concentration) as a cAMP binding protein. cAMP standards were run in parallel at serial concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 pmol/50 µl. After a two-hour incubation at 4° C, activated charcoal (26 mg/ml) (Norit, The Netherlands) was added to adsorb free cAMP. The mixture was then centrifuged, and 200 µl of supernatant was counted in EcoLite (ICN Pharmaceuticals, Costa Mesa, CA) scintillation fluid.

Pertussis toxin pretreatment of cells assayed for cAMP

hDOR/CHO and/or hDOR/$G_{\alpha i}$/CHO cells were pretreated for 18 – 24 hours with either serum-free IMDM or 50 – 75 ng/ml pertussis toxin (List Biological, Campbell, CA) in serum-free IMDM. For experiments involving the superactivation pretreatment protocol, either IMDM or 100 nM SNC80 in IMDM was added to the appropriate wells for the final four hours of pertussis toxin pretreatment. Cells were then washed once with fresh IMDM before the cAMP formation assay as described above.

Inositolphosphate (IP_1) Formation (adapted from (Berridge and Irvine, 1984))

In 24-well plates, growth medium was aspirated and replaced with serum-free IMDM containing 0.2 μM [^{3}H]-myo-inositol (Perkin Elmer Life Sciences, Boston, MA) for 18 hours in a humidified incubator at 37° C (5% CO_2). The cells were washed in 1 ml fresh IMDM for one hour. The medium was then replaced with IMDM containing 10 mM LiCl (Sigma Chemical, St. Louis, MO) for 10 minutes. Concentrations of SNC80 in IMDM were added for one hour, then replaced with 0.5 ml ice-cold methanol to terminate the reaction. The wells were scraped and the contents were transferred to a chloroform water mixture (1 ml and 0.5 ml, respectively) for inositol phosphate extraction. Following centrifugation, the extracts were loaded onto AG1-X8 resin (formate form) (BioRad, Richmond, CA) and washed three times with water followed by three washes with a solution of sodium tetraborate and formic acid (5 mM and 60 mM, respectively) to elute free inositol. IP_1 was then eluted with a solution of formic acid and ammonium formate (0.1 M and 0.2 M, respectively). Eluent was counted in 16 ml EcoLite scintillation fluid.

Pertussis toxin pretreatment of cells assayed for IP_1

In IP_1 formation experiments utilizing pertussis toxin, hDOR/CHO cells were pretreated with either 0.2 μM [^{3}H]-myo-inositol in serum-free IMDM or 0.2 μM [^{3}H]-myo-inositol with 50 ng/ml pertussis toxin in IMDM for the 18 hour loading phase. Cells were then washed once and assayed for IP_1 formation as described above.

Whole-cell Radioligand Binding Assay

In 24-well plates prepared as described above, growth medium was aspirated and washed once with 1 ml serum-free IMDM (37°C). The IMDM was replaced with 1 ml of various concentrations of [^{3}H]-naltrindole with and without 1 μM naloxone (non-specific binding). The plates were incubated in a humidified incubator at 37° C (5% CO_2) for 3 hr. The reaction was stopped by placing the plates on ice and washing the cells with 1 ml of ice-cold IMDM for 10 min. The IMDM was then aspirated and replaced with 0.5 ml of room temperature 1% Triton X-100 (Sigma Chemical, St. Louis, MO) and allowed to dissolve the cells for 30 min. The well contents were then transferred into scintillation vials and counted in 8 ml of Ecolite.

Data Analysis

Data were analyzed using Prism v3.02 (GraphPad, San Diego, CA) and are represented as mean ± standard error. To fit the data to a sigmoidal dose-response relationship, the following equation was used: $Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{(LogEC_{50} - X)(Hillslope)}}$ where X is the logarithm of the drug concentration and Y is the response. To fit the data to a saturation binding plot, the following equation was used: $Y = \frac{(B_{max})(X)}{K_d + X}$ where X is the concentration of radioligand and Y is the specifically bound radioligand. Statistical differences were ascertained by the student's t-test on the regression variables (top, bottom, $logEC_{50}$, etc.) as determined by the non-linear regression analysis where the Hill coefficient was fixed to unity. In the inhibition of forskolin-stimulated cAMP formation

experiments, results were normalized to 100% in the absence of SNC80 (top of the curve). In the adenylyl cyclase superactivation experiments, the overshoot in each cell line (comparison of the two curves representing each cell line pretreated with either IMDM or SNC80) was normalized to 100% of maximal cAMP formation in the IMDM-pretreated cells (top of the curve for IMDM-pretreated cells). Results of the IP_1 formation experiments are normalized to 100% of basal IP_1 formation.

Concomitant agonist and naltrindole pretreatment of hDOR/CHO cells

hDOR/CHO were plated and grown for two days in 24-well plates according to the GENERAL METHODS section. On the day of the experiment, the growth media was replaced with serum-free IMDM containing the δ-opioid agonist and varying amounts of naltrindole, depending on the assay. The cells were then assayed for cAMP formation.

Reverse transcriptase polymerase chain reaction (RT-PCR) with mRNA isolated from Chinese hamster ovary and mouse fibroblast B82 cells (from (Varga, et al., 2000))

Total messenger RNA from CHO or mouse fibroblast B82 cells was isolated according to the Micro-FastTrack mRNA Isolation Kit (Invitrogen, Carlsbad, CA). Briefly, hDOR/CHO cells were plated in a 60 mm polystyrene dish (Corning (Costar), Acton, MA) and grown to confluence in a 37° C humidified incubator (5% CO_2). Growth medium was aspirated and the cells were washed once with phosphate-buffered saline (PBS, containing 1 mM monopotassium phosphate, 155 mM sodium chloride, and 3 mM disodium phosphate). The cells were dislodged in 1 ml PBS, transferred to a microfuge

tube, and pelleted by centrifugation. The supernatant was replaced with 1 ml of 45° C 'SDS Lysis Buffer' (0.2 M sodium hydroxide and 1% sodium dodecyl sulfate) and the cells were resuspended. The NaCl concentration was adjusted to 0.5 M. The precipitated DNA was sheared by passing the lysate through a 21-gauge needle four times. One Oligo (dT) cellulose tablet was added to the lysate and allowed to disperse for 20 min. The cellulose was pelleted by centrifugation and the supernatant was replaced with 1.3 ml 'Binding Buffer.' The cellulose was pelleted again and was washed with 'Binding Buffer' three times. The final suspension was centrifuged in a spin-column/microfuge tube set. The cellulose was washed three times with 'Binding Buffer' (until the OD_{260} of the flow-through was less than 0.05). rRNA was removed with two 'Low Salt Wash Buffer' washes. Poly-A mRNA was eluted with two 100 µl 'Elution Buffer' washes. The mRNA was then precipitated in sodium acetate and ethanol (-70° C), centrifuged to form a pellet, then resuspended in 5 µl 'Elution Buffer.' The RNA yield was determined using the DNA DipStick® Kit (Invitrogen). RT-PCR was accomplished using primers directed towards conserved regions in the pertussis toxin-sensitive G-protein α subunit genes or adenylyl cyclase genes. E. coli transformed with the PCR products were grown and their DNA isolated for sequencing. The resulting sequences were compared to known genes in the Genbank online database for identity determination.

RESULTS

We initially investigated the human δ-opioid receptor density in intact cells by measuring [^{3}H]-naltrindole saturation binding (**Figure 5**). While demonstrating a dissociation constant (K_d) of 836 ± 157 pM, a figure which is slightly high, but still consistent with reported values (Yamamura, et al., 1992), hDOR/CHO cells exhibited a B_{max} of 1831 ± 150 fmol/million cells (about 1 million receptors per cell).

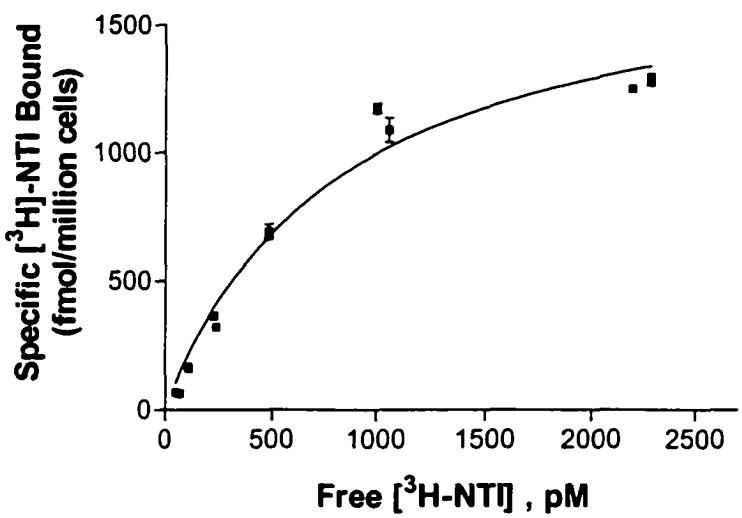

Figure 5: [^{3}H]-Naltrindole saturation binding in intact hDOR/CHO cells

[^{3}H]-naltrindole binding at 37° C was determined at equilibrium. The affinity constant (K_d) for naltrindole was found to be 836 ± 157 pM, and the human δ-opioid receptor B_{max} was 1831 ± 150 fmol/million cells (n = 2).

We examined δ-opioid receptor-mediated inhibition of adenylyl cyclase by a variety of agonists (**Figure 6**). SNC80 inhibited adenylyl cyclase to 6.2 ± 2% of control with an EC_{50} of 1.26 ± 0.37 nM (n=9). (-)TAN67 inhibited cAMP formation to 10.0 ± 2% of control with an EC_{50} of 0.56 ± 0.2 nM (n=2). Deltorphin II inhibited adenylyl cyclase

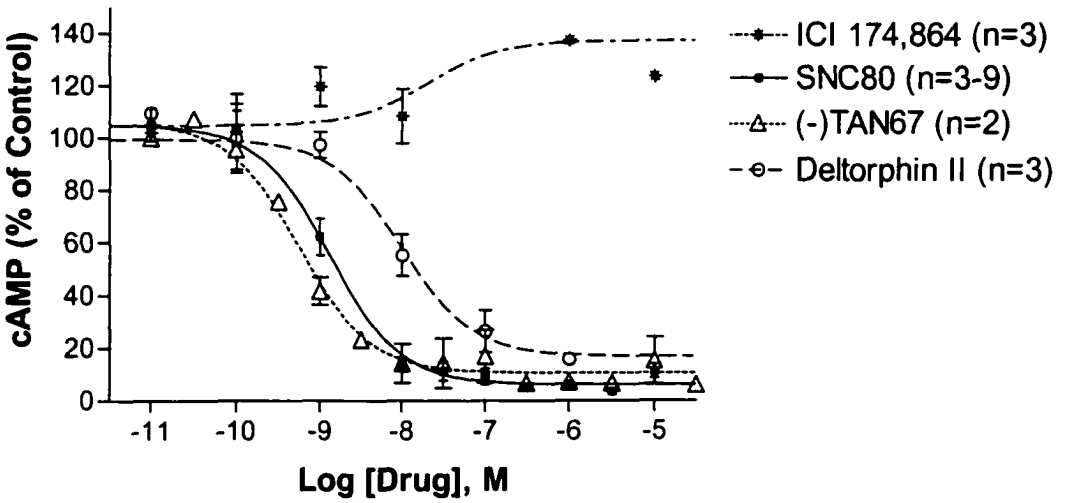

Figure 6: Modulation of Adenylyl Cyclase by Various Compounds

Each drug was tested for its ability to inhibit 100 μM forskolin-stimulated cAMP formation (10 μM forskolin in the ICI 174,864 experiment). The values for maximal inhibition by SNC80 (93.8 ± 2%), (-)TAN67 (90 ± 2%), and deltorphin (83 ± 6%) were not significantly different from one another. The EC_{50} values for SNC80 (1.26 ± 0.37 nM) and deltorphin (10.0 ± 7 nM) were not significantly different from one another, however (-)TAN67 was significantly more potent (EC_{50} = 0.56 ± 0.2 nM). ICI 174,864 significantly increased forskolin-stimulated cAMP formation by 137 ± 10% with an EC_{50} of 21 ± 90 nM.

to 17 ± 6% of control with an EC_{50} of 10.0 ± 7 nM (n=3). Additionally, we examined inverse δ-opioid agonism in this system by ICI-174,864. ICI 174,864 significantly increased forskolin-stimulated cAMP formation to 137 ± 10% of control ($p<0.01$) with an EC_{50} of 21 ± 90 nM (n=3).

SNC80-mediated inhibition of forskolin-stimulated cAMP formation was sensitive to pertussis toxin (**Figure 7**). Whereas SNC80 inhibited adenylyl cyclase to 4.1 ± 5% of control with an EC_{50} of 1.67 ± 1.3 nM, 50 ng/ml pertussis toxin treated cells showed no measurable inhibition.

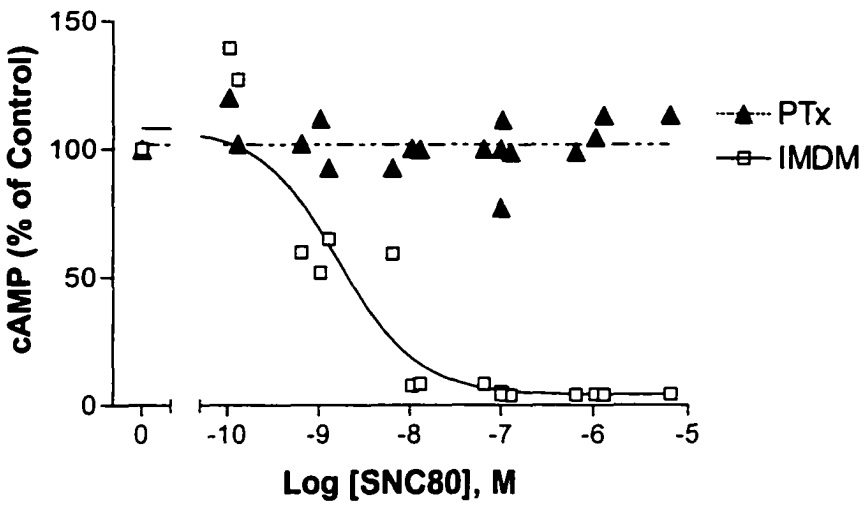

Figure 7: Pertussis toxin-sensitivity of SNC80-mediated adenylyl cyclase inhibition

Pertussis toxin pretreatment completely abolishes SNC80-mediated inhibition of 100 μM forskolin-stimulated cAMP formation. Whereas SNC80 inhibits IMDM-pretreated cells by 96 ± 5% with an EC_{50} of 1.67 ± 1.3 nM, there is no measurable inhibition after 18-24 hours with 50 ng/ml pertussis toxin (n = 4).

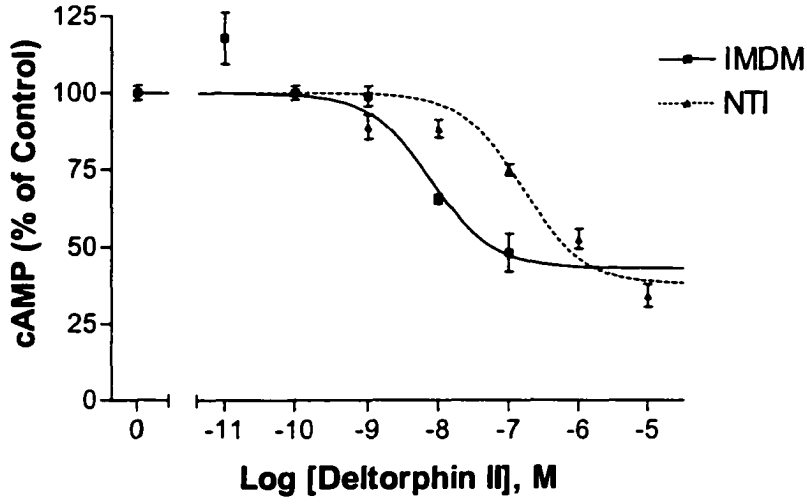

Figure 8: Naltrindole antagonism of deltorphin-mediated adenylyl cyclase inhibition

Naltrindole (NTI) competitively antagonized deltorphin-mediated inhibition of 100 μM forskolin-stimulated cAMP formation. The EC_{50} of deltorphin in the absence of NTI was 7.74 ± 11 nM, whereas 100 nM NTI shifted this value to 153 ± 120 nM. The E_{max} values in the absence and presence of NTI were not significantly different (43 ± 9% and 38 ± 5%, respectively).

δ-opioid agonist-mediated inhibition of forskolin-stimulated cAMP formation was competitively inhibited by 100 nM naltrindole. Due to the use of several naltrindole concentrations and the choice of δ-opioid agonist, **Figure 8** is a representative example of naltrindole's effect. In this experiment, deltorphin II inhibited 10 μM forskolin-stimulated cAMP formation with an E_{max} of 43 ± 9% of control and an EC_{50} of 7.74 ± 11 nM. In the presence of 10 nM naltrindole, deltorphin's E_{max} was 38 ± 5% of control with an EC_{50} of 153 ± 120 nM. Whereas the E_{max} values were not significantly different ($p>0.5$), the EC_{50} values were ($p<0.01$). The affinity of the antagonist was calculated

using the Schild equation as follows: $K_e = \frac{[antagonist]}{DoseRatio - 1}$ where $DR = \frac{[EC_{50}]_{ant}}{[EC_{50}]}$. The K_e for naltrindole was determined (using the EC_{50} values to derive the dose ratio) to be 0.53 ± 0.3 nM.

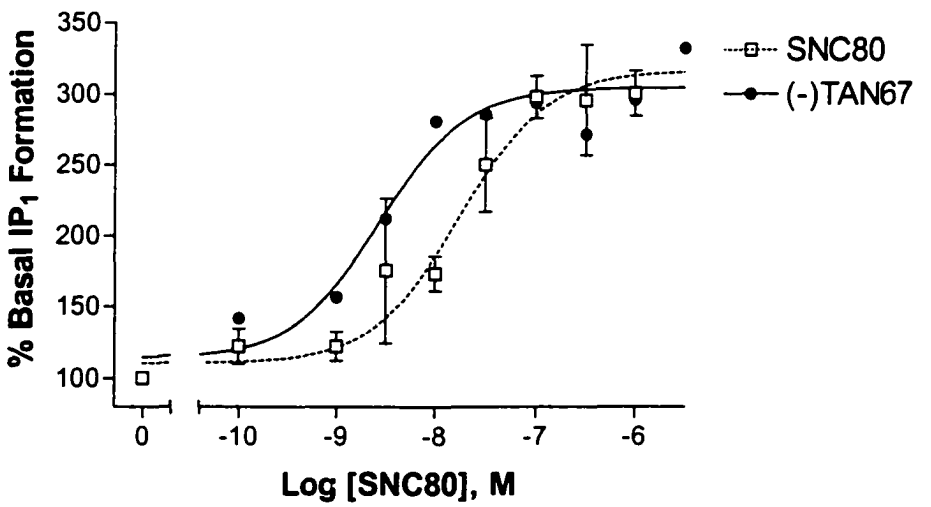

Figure 9: δ-opioid agonist-mediated PLCβ stimulation in hDOR/CHO cells

SNC80 stimulated IP_1 formation by 317 ± 11% of basal and an EC_{50} of 17.8 ± 10 nM (n=12), whereas (-)TAN67 stimulated with an E_{max} of 305 ± 8% of basal and an EC_{50} of 2.7 ± 2 nM (n=1).

The ability of the human δ-opioid receptor in CHO cells to couple to phospholipase Cβ activation was examined. As discussed in the GENERAL METHODS, PLCβ activation was measured by isolating accumulated [^{3}H]-inositol monophosphate (IP_1) in the presence of lithium. SNC80 and (-)TAN67 were examined for their abilities to stimulate IP_1 accumulation (**Figure 9**). In this assay, SNC80 stimulated IP_1 formation

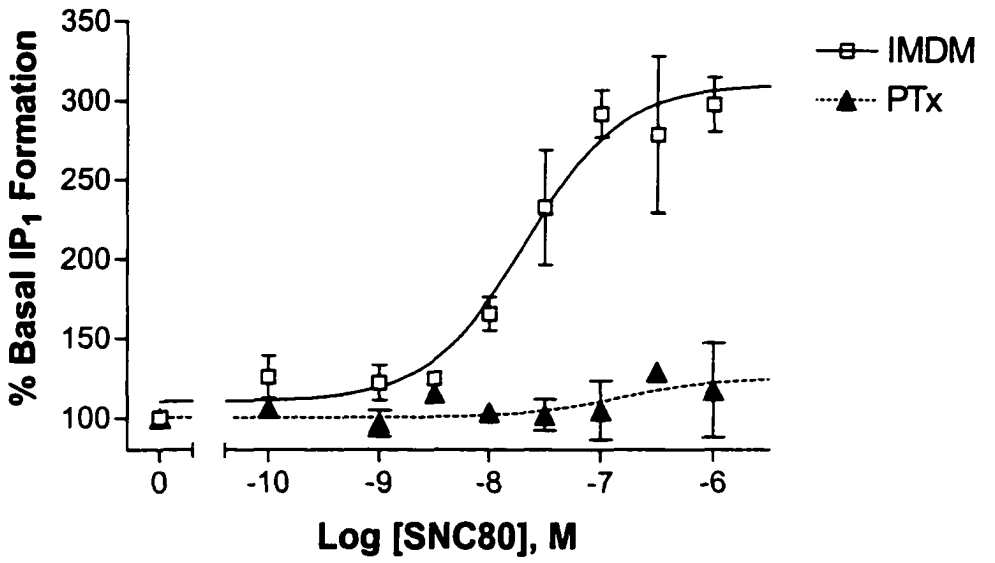

Figure 10: Pertussis toxin-sensitivity of SNC80-mediated PLCβ stimulation SNC80 stimulated IP_1 formation by 311 ± 12% of basal and an EC_{50} of 20.9 ± 13 nM (n=11), whereas cells treated with 50-75 ng/ml pertussis toxin for 18-24 hours showed no measurable IP_1 formation (n=4).

with an E_{max} of 317 ± 11% of basal and an EC_{50} of 17.8 ± 10 nM (n=12), whereas (-)TAN67 stimulated with an E_{max} of 305 ± 8% of basal and an EC_{50} of 2.7 ± 2 nM (n=1). While there was no statistically significant difference in their intrinsic activities, the EC_{50} of SNC80 appeared to be significantly higher ($p<0.01$).

SNC80-mediated PLCβ activation was also shown to be pertussis toxin sensitive (**Figure 10**) (Rubenzik, et al., 2001). By pretreating hDOR/CHO cells with 50-75 ng/ml pertussis toxin along with the [^{3}H]-myoinositol, no SNC80-mediated IP_1 formation was detected, whereas untreated cells demonstrated an SNC80-stimulated activity of 311 ± 12% of basal and an EC_{50} of 20.9 ± 13 nM (n=11). The basal PLCβ activity in

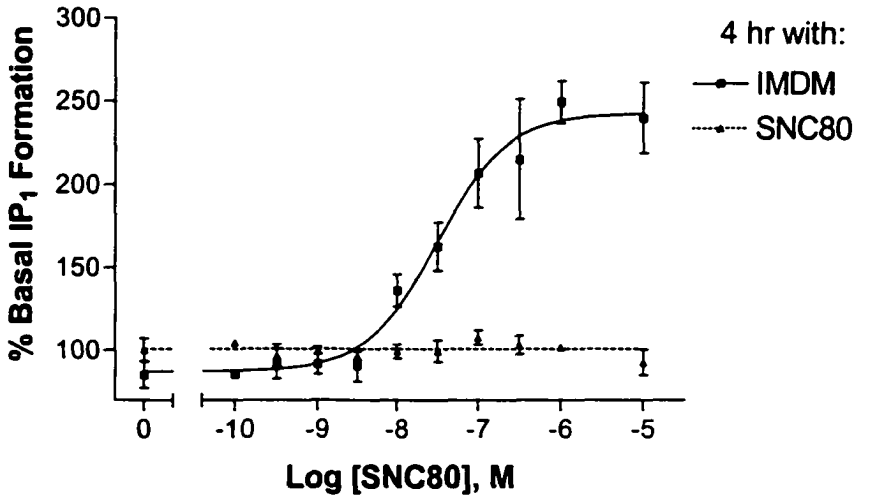

Figure 11: SNC80-mediated PLCβ stimulation after chronic SNC80 treatment

SNC80 stimulated IP_1 formation by 243 ± 9% of basal with an EC_{50} of 30.9 ± 19 nM (n = 4). Following a 4 hour pretreatment with 100 nM SNC80, SNC80 showed no measurable IP_1 formation (n = 4).

hDOR/CHO cells was 589 ± 900 CPM (n=11, data not shown).

In addition to studies utilizing acutely applied agonists, we investigated the ability of hDOR/CHO cells treated chronically with SNC80 to couple to PLCβ. This was done by pretreating the cells under exactly the same conditions as those that result in adenylyl cyclase superactivation, but follow with an SNC80-mediated IP_1 formation assay (**Figure 11**). In these experiments, 4 hour IMDM pretreatment resulted in an SNC80-mediated PLCβ stimulation of 243 ± 9% of basal with an EC_{50} of 30.9 ± 19 nM. Following a 4 hour pretreatment with 100 nM SNC80, SNC80 showed no measurable IP_1 formation.

After demonstrating that all of the acute δ-opioid receptor-mediated responses in Chinese hamster ovary cells examined here were directed through pertussis toxin-sensitive G-proteins, we defined the G-protein $\alpha_{i/o}$ subunit expression profile in these cells. Using the reverse transcriptase polymerase chain reaction with mRNA purified from hDOR/CHO cells as template and primers directed toward conserved G_i/G_o regions, we found expression of $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha oA/B}$, and $G_{\alpha t1}$ (retinal rod transducin) (**Table 1**) (Varga, et al., 2000).

	CHO	B82
$G_{\alpha i2}$	+	+
$G_{\alpha i3}$	+	+
$G_{\alpha oA/B}$	+	+
$G_{\alpha t1}$	+	-

Table 1: Pertussis toxin-sensitive Gα subunit isoforms in Chinese hamster ovary and mouse fibroblast B82 Cells

DISCUSSION

The human δ-opioid receptor density in hDOR/CHO cells appears to have increased by about 10-fold over the years of selection in cell culture. As predicted, however, measurable changes in function, such as receptor-mediated adenylyl cyclase inhibition, [^{35}S]-GTPγS binding, and even down-regulation have not occurred due to the vast receptor reserve. The ratio of receptors to G-proteins was sufficiently large when the

cell line was created that the 10-fold rise in expression has not resulted in noticeable differences.

A generalized scheme of the G-protein cycle is illustrated in **Figure 1**. In this cycle, human δ-opioid receptor activation stimulates GDP-GTP turnover in $G_{\alpha i/o}$ G-proteins. Normally, the $G_{\alpha i/o}$ hydrolyzes its bound GTP to GDP so that the $G_{\alpha i/o}$ can recombine with $G_{\beta\gamma}$ to reset the cycle for another round. By radiolabeling a less hydrolysable form of GTP, [^{35}S]-GTPγS was created. When introduced into the cellular environment, [^{35}S]-GTPγS associates with activated $G_{\alpha i/o}$, and halts the cycle at this point. By collecting the membranes through vacuum filtration, the amount of bound [^{35}S]-GTPγS can be measured and correlated with $G_{\alpha i/o}$ receptor activation.

Our laboratory has measured the ability of the human δ-opioid receptor in CHO cells to couple to endogenously expressed G-proteins by way of [^{35}S]-GTPγS binding experiments (Quock, et al., 1997). In these studies, it was demonstrated that potent δ-opioid-mediated G-protein activation occurs using a variety of δ-opioid agonists (SNC80, pCl-DPDPE, and (-)TAN67). This response was pertussis toxin-sensitive, as measured by SNC80-induced [^{35}S]-GTPγS binding in the presence of pertussis toxin (Varga, et al., 2000).

Much like δ-opioid receptor coupling to G-protein activation, the demonstration that the receptor couples to adenylyl cyclase inhibition is a well-established opioid response in a diverse array of tissues (Sharma, et al., 1975a; Knapp, et al., 1995; Murthy and Makhlouf, 1996; Nevo, et al., 1998). Therefore, it is important for any applicable

opioid expression system to exhibit this characteristic. We were able demonstrate human δ-opioid receptor coupling to modulation of adenylyl cyclase in Chinese hamster ovary cells by a number of structurally different δ-opioid agonists (peptidic, deltorphin II and ICI 174,864 and non-peptidic, SNC80 and (-)TAN67). Furthermore, this inhibitory response was shown to be pertussis toxin- and naltrindole-sensitive, indicating that it was indeed mediated by the human δ-opioid receptor through $G_{i/o}$ G-proteins. The demonstration of inverse agonism by ICI 174,864 was very interesting. This δ-opioid ligand, billed originally as a selective antagonist, has been shown in a number of systems to behave as a δ-opioid inverse agonist. Displaying its inverse agonist properties in hDOR/CHO cells speaks to the robustness and sensitivity of the hDOR/CHO cells as a model system. These results validate the hDOR/CHO expression system as a physiologically relevant model for studying the human δ-opioid receptor.

The ability of the human δ-opioid receptor to couple to phospholipase Cβ (PLCβ) represents a common, though by no means universal, opioid response (Tsu, et al., 1995; Murthy and Makhlouf, 1996; Rubenzik, et al., 2001). Since this response was shown to be pertussis toxin-sensitive, we could safely conclude that PLCβ activation is also mediated through the same pool of $G_{i/o}$-proteins that inhibit adenylyl cyclase. The activation of PLCβ by many $G_{\alpha i/o}$-coupled receptors signals through the release of $G_{\beta\gamma}$. This facet was experimentally important, as we were able to utilize the IP_1 accumulation assay as a measure of $G_{\beta\gamma}$-mediated signaling. However, the physiological importance of the PLCβ pathway is underscored by its ultimate activation of a very broad array of

enzymes and chemical mediators. It is understood that calcium transients measured in response to opioid receptor stimulation occur through activation of this pathway (Fukuda, et al., 1996). The phospholipase C family hydrolyzes phosphatidylinositol bisphosphate (PIP_2) to form inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with the heparin-sensitive IP_3 receptors on the endoplasmic reticulum to cause the release of calcium. This intracellular calcium is important for the activation of various effectors, such as certain protein kinase C (PKC) isoforms (the "classical" PKCs, which require calcium), and calcium-calmodulin protein kinases, as well as countless other enzymes modulated by available calcium. The liberated DAG activates the "novel" PKCs and, in conjunction with calcium, is also required to activate the "classical" PKCs.

Much like opioid receptor-mediated PLCβ activation, the process of transactivation of growth factor tyrosine kinase receptors requires $G_{\beta\gamma}$. And $G_{\beta\gamma}$ is responsible for the opening of inwardly rectifying potassium channels (in excitable tissues). On the whole, signal transduction through liberated $G_{\beta\gamma}$ amounts to a large portion, if not the majority, of the ultimate signals mediated by G-protein-coupled receptors and opioid receptors in particular. Therefore, it was important for us to be able to measure the intensity of these responses indirectly through the IP_1 formation assay.

Initially, the ability of SNC80 to inhibit forskolin-stimulated cAMP formation in hDOR/CHO cells was reported by our laboratory in 1995. At that time, Malatynska et al. (1996) demonstrated the desensitization that occurs at the receptor level in hDOR/CHO cells treated chronically with the δ-opioid agonist, SNC80. The fascinating results from this experiment were dependent upon how the data were plotted. When graphed as a

percent of maximally forskolin simulated control, the curves originated together, but the cells pretreated with SNC80 did not inhibit cAMP formation as effectively as the control cells. This looked like typical desensitization of the adenylyl cyclase inhibition response with SNC80-pretreated cells exhibiting a lower intrinsic activity. However, when plotted as raw pmol cAMP per million cells, the pretreated cells exhibited a cAMP overshoot that was inhibited by SNC80 down to pretreatment levels (**Figure 12**).

When represented as such, this experiment provides an explanation for what the hDOR/CHO cells do when they superactivate adenylyl cyclase: In the continued inhibitory influence of the chronic δ-opioid agonist, the cells compensate by increasing the activity of the adenylyl cyclase to exactly match the inhibition. When the cells are pretreated with SNC80, it is with a maximally stimulating concentration- the far right

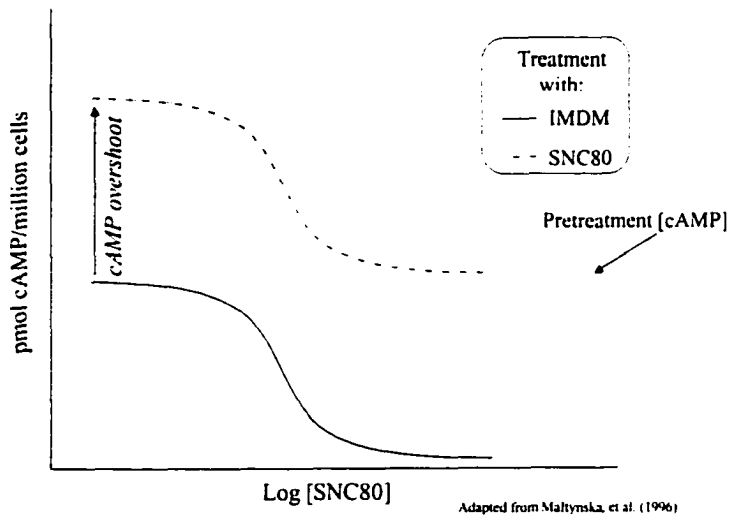

Figure 12: Diagram of inhibition of adenylyl cyclase after superactivation

SNC80 pretreatment and washout results in an increased forskolin-stimulated adenylyl cyclase activity and a cAMP overshoot. While adenylyl cyclase is superactivated, SNC80 inhibits it to pretreatment levels. This indicates that adenylyl cyclase superactivation is the result of the cells compensating for the acute inhibition caused by SNC80.

region of the concentration-response curve. In **Figure 12**, the pretreated cells are inhibited maximally by SNC80 right down to a cAMP concentration exactly matching their concentrations in the chronic presence of maximally inhibiting SNC80. When plotted as a percent of control, the data become prone to misinterpretation as simply desensitization, thereby ignoring one of the most substantial results from such experiments, which is the influence of adenylyl cyclase superactivation.

Our results from the desensitization of the PLCβ response after chronic SNC80 were not nearly as complex. The basal raw IP_1 formation in SNC80 pretreated hDOR/CHO was not different from untreated cells (in CPM for each independent experiment, data not shown). The fact that this response was completely shut off by pretreatment conditions identical to those that cause adenylyl cyclase superactivation indicated to us that even if the PLCβ pathway was important for the formation of adenylyl cyclase superactivation, it must not have been vital for maintaining it. In one brief attempt to investigate the mechanism of this PLCβ negative feedback, I measured SNC80-mediated IP_1 formation in the presence of 8-bromo-cAMP, a cell-permeable analog of cAMP capable of stimulating PKA. The rationale behind this experiment was derived from the presence of extraordinarily high concentrations of intracellular cAMP following washout of the SNC80 in the chronic treatment paradigm (the cAMP overshoot). We thought it possible that this spike in cAMP could activate PKA to negatively modulate PLCβ. The results were negative (data not shown), indicating to us that the intracellular cAMP concentration did not modulate receptor-mediated PLCβ activity. Still, this experiment was important for indicating, as other groups have

demonstrated, that the feedback that causes adenylyl cyclase superactivation does not rely on intracellular cAMP concentrations, in spite of the fact that the cell always compensates for the exact extent of adenylyl cyclase inhibition.

Finally, we felt that knowledge of the signal transduction mediators used by the human δ-opioid receptor in CHO cells would facilitate our understanding of how adenylyl cyclase superactivation occurs in these cells. It was no surprise to detect $G_{\alpha i2}$, $G_{\alpha i3}$, and $G_{\alpha o}$, but the discovery of $G_{\alpha t}$ was unusual as this G-protein is traditionally thought to occur in retinal rod cells, an excitable tissue. We were so fascinated by its presence that others in the laboratory created a pertussis toxin-insensitive mutant of $G_{\alpha t}$ and created a CHO cell line that expressed both the human δ-opioid receptor and this mutant $G_{\alpha t}$ (hDOR/Gαt/CHO). They were able to demonstrate coupling of the human δ-opioid receptor to $G_{\alpha t}$ by measuring SNC80-mediated [^{35}S]-GTPγS binding in cell membranes (increased maximal [^{35}S]-GTPγS binding or residual maximal [^{35}S]-GTPγS binding after pertussis toxin pretreatment) (Varga, et al., 2000). However, it is important to note that the receptor reserve in these cells allowed the human δ-opioid receptor to couple with equal quantities to $G_{\alpha i/o}$ G-proteins in both the presence and absence of $G_{\alpha t}$. This factor will become significant in subsequent chapters.

In conclusion, we have characterized the human δ-opioid receptor in Chinese hamster ovary cells using a number of different endpoints. We quantified expression with [^{3}H]-naltrindole binding, examined the coupling of the receptor to adenylyl cyclase and PLCβ, and shown these signals to be transmitted through $G_{\alpha i/o}$. Furthermore, we

discovered the desensitization of the PLCβ response after chronic SNC80 pretreatment in hDOR/CHO cells. Overall, hDOR/CHO cells are a sensitive and relevant model system for studying the human form of the δ-opioid receptor *in vitro*.

CHAPTER 3: CHARACTERIZATION OF ADENYLYL CYCLASE SUPERACTIVATION IN hDOR/CHO CELLS

INTRODUCTION

While working in Dr. Yamamura's laboratory, Ewa Malatynska (Malatynska, et al., 1996) demonstrated a forskolin-stimulated cAMP overshoot in hDOR/CHO cells in 1996 much like the NG108-15 cell data obtained by Nirenberg and his coworkers in 1975 (Sharma, et al., 1975a). This finding created an opportunity to investigate the molecular mechanism of the cAMP overshoot in novel ways, utilizing the human form of the δ-opioid receptor and expressing it in Chinese hamster ovary cells. SNC80 was chosen as the agonist in these assays due to its high potency, intrinsic activity, and relative water solubility (allowing it to be washed from cell culture). Our general hypothesis for this project was to demonstrate that CHO cells stably expressing the human δ-opioid receptor were a relevant and robust system for investigating the cellular processes that produce adenylyl cyclase superactivation. To accomplish this, we set out to characterize some of the fundamental features of this phenomenon in hDOR/CHO cells.

The characterization of this cell line not only included acute agonist treatment as described in the previous chapter, but also more chronic treatment to investigate the fate of important signal transduction systems upon tonic human δ-opioid receptor stimulation. Under these conditions, we investigated the adenylyl cyclase system, the phospholipase Cβ system, and others in the laboratory observed changes in human δ-opioid receptor expression and G-protein coupling.

We first characterized adenylyl cyclase superactivation. It was imperative to verify that the agonist-mediated adenylyl cyclase superactivation was δ-opioid antagonist sensitive, indicating that the development of superactivation was dependent on δ-opioid receptor activation. The accepted method for demonstrating this has always been to show reversibility or abolishment of the response by treatment with a selective antagonist if one is available. In these experiments, we used the extraordinarily high affinity antagonist, naltrindole, which typically has a K_d of about 50 pM at the δ-opioid receptor.

The next detail involved human δ-opioid receptor coupling to endogenously expressed G-proteins in the Chinese hamster ovary (CHO) cell environment. Many cellular signals downstream of $G_{i/o}$-protein-coupled receptors have been shown to be pertussis toxin-insensitive, effectively bypassing G-protein activation. An example in the δ-opioid receptor field would be the pertussis toxin-insensitive agonist-mediated receptor down regulation in hDOR/CHO cells or C6 glial cells expressing the human μ-opioid receptor (Yabaluri and Medzihradsky, 1997). Knowledge of pertussis toxin insensitivity changes how the data would be interpreted. Because such signal transduction was not impossible, we determined the pertussis toxin-sensitivity of adenylyl cyclase superactivation by pretreating the cells with a supramaximal concentration of pertussis toxin overnight before examining the SNC80-induced cAMP overshoot.

One important factor to consider in chronic treatment paradigms is the regulation of gene expression because of such treatment. Upregulation of adenylyl cyclase isoforms would be a sensible response to tonic inhibition of the adenylyl cyclase due to δ-opioid receptor activation. Furthermore, expression of other proteins, such as $G_{\alpha s}$ or $G_{\alpha s}$-coupled

receptors, could positively modulate the activity of adenylyl cyclase and compensate for a chronic inhibition. A simple method for determining the contribution of such putative responses was to treat the cells with cycloheximide, an inhibitor of novel protein synthesis. We were not expecting to find any sensitivity of the cAMP overshoot to cycloheximide because, out of the many people working in this field, few groups have been able to demonstrate the requirement for protein upregulation. Rivera and Gintzler showed that, in a μ-opioid expressing myenteric muscle plexus preparation, upregulation of ACIV occurred in response to chronic morphine treatment (Rivera and Gintzler, 1998). But Avidor-Reiss et al. demonstrated a cycloheximide-insensitive cAMP overshoot in CHO cells stably expressing the μ-opioid receptor (Avidor-Reiss, et al., 1995), and the original cAMP overshoot published by Sharma and coworkers was reduced only very modestly by cycloheximide treatment (Sharma, et al., 1977). Because our hDOR/CHO system was so closely related to the μ-opioid-expressing CHO cells and did not contain any ACIV (as will be discussed below), we expected our results to reflect those of Avidor-Reiss et al.'s (1995).

Next, we examined the time-course of SNC80-mediated adenylyl cyclase superactivation. The amount of time required for superactivation to develop has been rather difficult to reconcile. Most opioid receptor responses that have been investigated, including agonist-mediated release of intracellular calcium, typical acute effector activation, and even desensitization of these responses, appear to occur in seconds or minutes, not hours. Adenylyl cyclase superactivation seemed to run counter to this general observation. For example, a short-term agonist pretreatment, such as the 20

minutes used to acutely inhibit adenylyl cyclase, does not result in any increase in forskolin-stimulated cAMP formation. Based on earlier work by others in the laboratory, we knew that the same twenty-four hours it took to maximally down-regulate the human δ-opioid receptor also produced a cAMP overshoot, but we did not know what the time course really might have been, whereas the down-regulation time-course was previously published (Malatynska, et al., 1996). Our experience and the results of others indicated that 4 to 5 hours were all that were required to induce maximal superactivation. In our attempts to definitively characterize the cAMP overshoot, we conducted an SNC80-mediated adenylyl cyclase superactivation time-course extending out to 5 hours. Because of these results, we were able to shorten the pretreatment time to 4 hours, with essentially equivalent results to the original 24-hour overshoot.

We also investigated the potency of SNC80 in inducing the cAMP overshoot. The rationale for this work extended from our interest in determining which pathway, $G_{\alpha i/o}$ or $G_{\beta\gamma}$ might be involved in adenylyl cyclase superactivation. Our standards were the higher potency $G_{\alpha i/o}$-mediated adenylyl cyclase inhibition and the lower potency $G_{\beta\gamma}$-mediated PLCβ activation. As outlined in the previous chapter, the EC_{50} for SNC80-mediated inhibition of forskolin-stimulated cAMP formation was 1.29 ± 0.038 nM, whereas PLCβ activation had an EC_{50} of 20.0 ± 13 nM, or about 15-fold less potent. Knowing the potency of SNC80 in mediating adenylyl cyclase superactivation might have indicated which pathway, $G_{\alpha i/o}$ or $G_{\beta\gamma}$, was more pertinent.

As stated previously, SNC80 was originally chosen for hDOR/CHO cell pretreatment due to its relative water solubility, allowing it to be washed from cells in

culture. Since then, we have explored the use of conformationally stable peptidic δ-opioid agonists due to their increased water solubility. The first, examined fortuitously, was D-pen^2-D-pen^5-enkephalin (DPDPE). DPDPE was created at the University of Arizona by the Victor Hruby, Ph.D. peptide synthesis chemistry group. Since its synthesis, it has been regarded as the standard against which other δ-opioid selective agonists are compared. The second agonist, D-ala^2-deltorphin II (deltorphin), is a highly selective δ-opioid agonist. It was originally isolated from frog skin, and then chemically modified to offer a more stable active conformation. We have found deltorphin II to have maximal intrinsic activity (equivalent to other agonists in the [^{35}S]-GTPγS binding assay) and acceptable chemical longevity in cell culture in spite of its peptidic nature.

RESULTS

Due to our continued interest in the use of hDOR/CHO cells for investigating adenylyl cyclase superactivation as a model for opioid tolerance and withdrawal, we initially reproduced the cAMP overshoot observed by Malatynska et al. (1996). The data were typically obtained as pmol of cAMP per 50 μl of assay volume, and then converted to pmol of cAMP per million cells based on the available counts (from cells plated and treated in parallel with the experimental cells). Despite the inconsistency in the data when combined as pmol cAMP per million cells, the cAMP overshoot was statistically significant when regressed this way. For unknown reasons, representing the data as pmol per million cells created considerable variability between experiments.

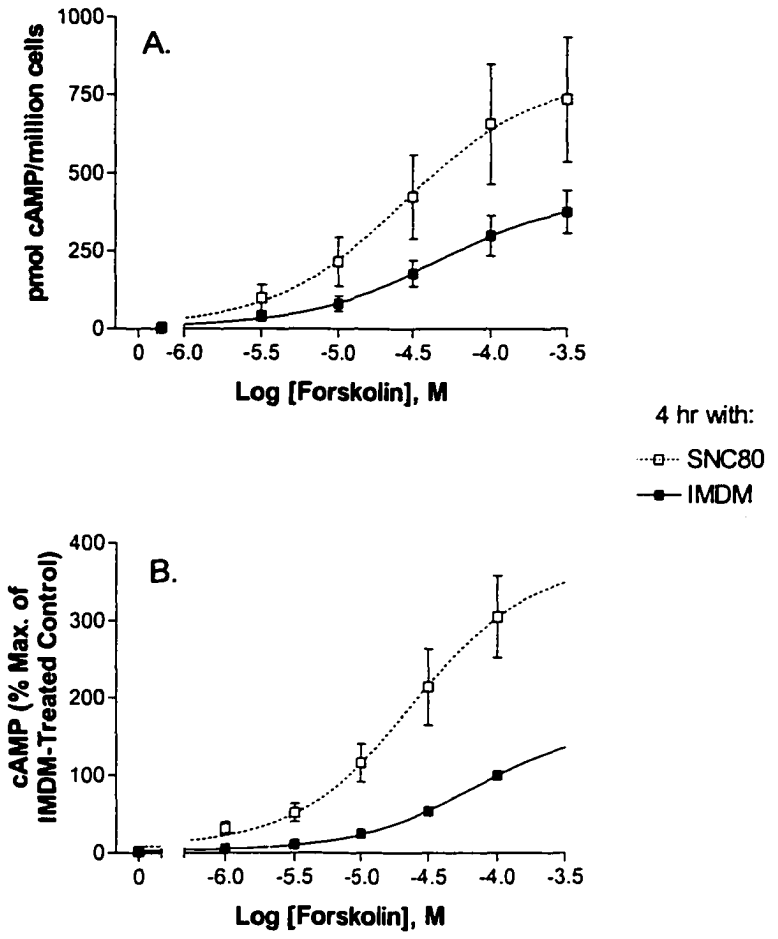

Figure 13: SNC80-mediated superactivation in hDOR/CHO cells

A. SNC80 pretreatment and washout results in an increased forskolin-stimulated adenylyl cyclase activity and a cAMP overshoot. Untreated cells exhibited an E_{max} of 428 ± 8.2 pmol per million cells, whereas 4 hour 100 nM SNC80 pretreated cells exhibited an E_{max} of 810 ± 19 pmol per million cells (n=5). **B.** When converted to a percent of maximally stimulated IMDM-treated cells, 100 nM SNC80 induced a cAMP overshoot of 376 ± 12 % of IMDM-treated cells (n=24).

In my hands, however, SNC80 produced a significant ($p<0.01$) cAMP overshoot of 810 ± 19 pmol per million cells after 4 hr 100 nM SNC80 versus 428 ± 8.2 pmol per million cells in IMDM-treated cells (n=5) (**Figure 13A**). However, to be able to combine multiple experiments for statistical analysis, we opted to express the results as a percent of maximally stimulated IMDM-treated control. When transformed in this manner, the data became highly consistent, producing a

cAMP overshoot of 376 ± 12 % of IMDM-treated cells (n=24) ($p<0.01$) (**Figure 13B**).

Pretreatment with both 100 nM SNC80 and 1 μM naltrindole blocked the cAMP overshoot as compared with hDOR/CHO cells pretreated with naltrindole alone (**Figure 14**). This figure is a representative example of three independent experiments. These results could not be combined because they were obtained at different times using either different agonists (SNC80 or deltorphin), or different concentrations of naltrindole, but the ability of naltrindole to prevent adenylyl cyclase superactivation was consistent. Naltrindole alone did raise the raw forskolin-stimulated cAMP formation value. In the raw data used to produce **Figure 14**, naltrindole-treated cells displayed a significant

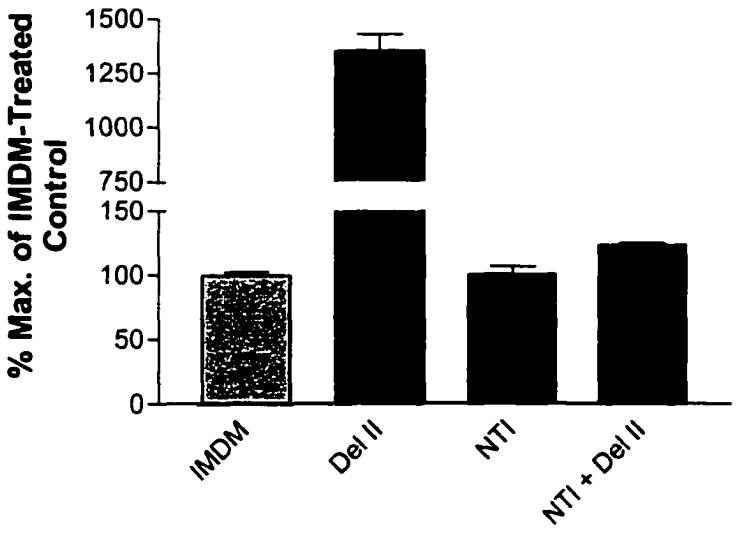

Figure 14: Naltrindole antagonism of deltorphin-mediated superactivation

In this representative experiment, deltorphin pretreatment and washout results in a forskolin-stimulated cAMP overshoot. The overshoot in each group (± NTI) was normalized to IMDM- (or NTI-) treated and forskolin-stimulated cells. In the absence of naltrindole (NTI), 4-hour deltorphin-pretreated cells exhibited a 100 μM forskolin stimulation of 1350 ± 77% of IMDM-treated cells. In the presence of 1 μM NTI, deltorphin pretreatment and washout resulted in an E_{max} of 123 ± 1% of NTI-treated cells.

increase from 1.2 ± 0.07 to 15 ± 1 pmol cAMP per 50 µl assay volume. However, even as pmol cAMP per 50 µl, hDOR/CHO cells pretreated with deltorphin II and naltrindole showed no significant cAMP overshoot ($p>0.05$). The data were re-plotted as a percent of maximally stimulated IMDM-treated control, thereby removing the influence of this rise. Under these conditions, naltrindole significantly attenuated deltorphin II-mediated adenylyl cyclase superactivation by 91 ± 2% (from 1350 ± 77 to 123 ± 1%), effectively eliminating the cAMP overshoot.

Many groups investigating opioid-induced adenylyl cyclase superactivation have determined that this phenomenon is pertussis toxin sensitive (Thomas and Hoffman, 1992; Avidor-Reiss, et al., 1995). The overshoot in hDOR/CHO cells was likewise shown to be pertussis toxin sensitive (**Figure 15**) (Rubenzik, et al., 2001). Treatment with pertussis toxin significantly lowered the raw forskolin stimulated cAMP formation from 853 ± 91 to 503 ± 28 pmol per million cells ($p<0.01$, $n=10$, data not shown), whereas the raw basal cAMP formation was not significantly different (3.52 ± 0.8 and 3.59 ± 0.8 pmol per million cells for untreated and pertussis toxin treated cells, respectively, $p>0.50$, $n=10$). As a percent of maximally stimulated control, 18-24 hr with 50-75 ng/ml pertussis toxin significantly attenuated the increase in forskolin-stimulated cAMP formation after 4 hours with 100 nM SNC80 (237 ± 21 and 262 ± 45% for untreated and SNC80-treated cells, respectively, $p>0.50$, $n=10$). As stated above, the transformation of the data in this way eliminated the differences in the forskolin-stimulated values between the untreated and pertussis toxin-treated hDOR/CHO cells. Whereas untreated hDOR/CHO cells exhibited a significant cAMP overshoot of 558 ± 97 ($p<0.01$), pertussis toxin treated cells

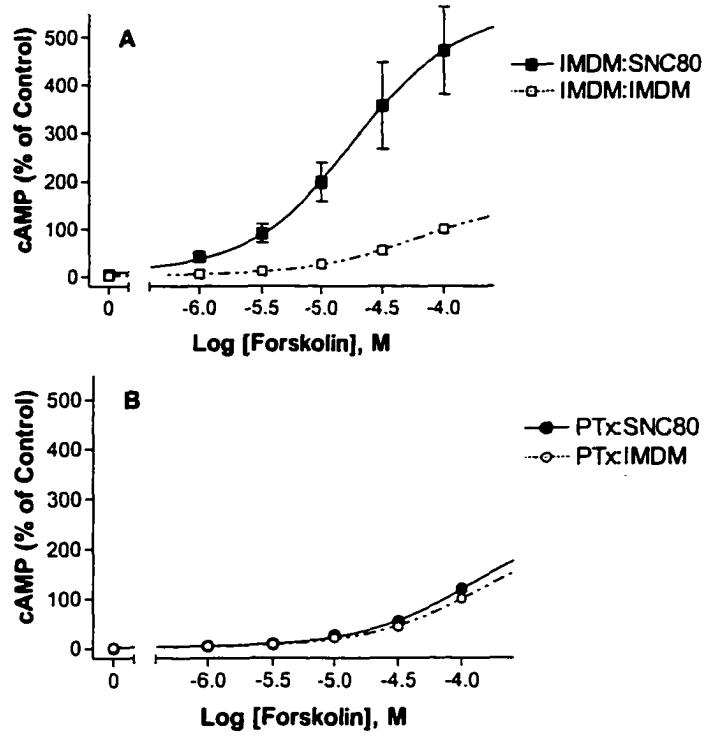

Figure 15: Pertussis toxin sensitivity of adenylyl cyclase superactivation

IMDM-treated cells exhibited a 100 nM SNC80-mediated cAMP overshoot of 558 ± 97% of maximally stimulated IMDM-treated control (n=10). After 18-24 hours with 50-75 ng/ml pertussis toxin, there was no significant cAMP overshoot noted (n=10, p>0.50).

showed a statistically insignificant 262 ± 45 % of maximally forskolin-stimulated controls (p>0.50).

Co-pretreatment with cycloheximide did not affect adenylyl cyclase superactivation by 100 nM SNC80. Although these were preliminary results, they were obtained using conditions demonstrated by others to effectively inhibit novel protein synthesis in CHO cells (Avidor-Reiss, et al., 1995). Much like the results from Avidor-Reiss, et al. (1995), hDOR/CHO cells pretreated with 10 μM cycloheximide exhibited a significantly reduced forskolin-stimulated cAMP formation. Whereas cells treated without cycloheximide (and without SNC80) exhibited a maximally forskolin-stimulated value of 418 ± 25 pmol cAMP per million cells, cycloheximide treated cells

made 207 ± 18 pmol cAMP per million cells. Cells treated without cycloheximide (and with SNC80) exhibited a maximally forskolin-stimulated value of 720 ± 63 pmol cAMP per million cells, whereas cycloheximide treated cells formed 382 ± 0.28 pmol cAMP per million cells. The cAMP overshoot both in the presence and absence of cycloheximide was significant ($p<0.01$), even when expressed as raw pmol per million cells.

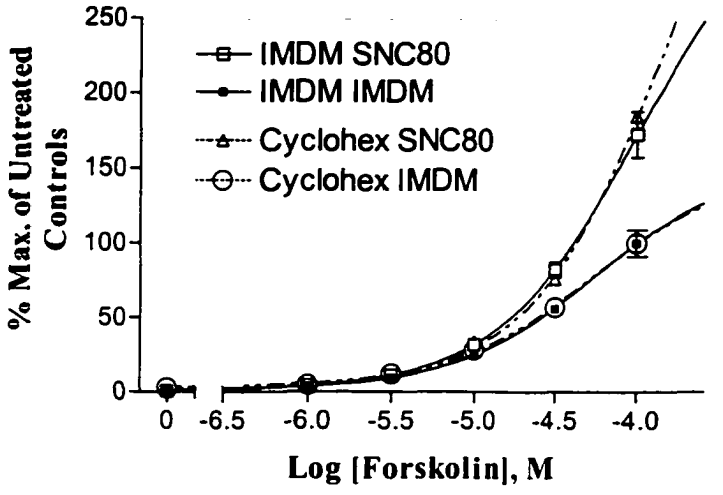

Figure 16: Cycloheximide insensitivity of adenylyl cyclase superactivation

In this representative experiment, IMDM-treated cells exhibited a 100 nM SNC80-mediated cAMP overshoot of 172 ± 15% of maximally stimulated IMDM-treated control (100 μM forskolin). After 18-24 hours with 10 μM cycloheximide, the SNC80-mediated cAMP overshoot was 184 ± 0.13%.

However, when the data were transformed as a percent of maximally forskolin-stimulated IMDM-treated control, it became obvious that cycloheximide had no affect on the ability of these cells to superactivate adenylyl cyclase (**Figure 16**). Cells not treated with cycloheximide exhibit a cAMP overshoot of 172 ± 15% of control, whereas cycloheximide treated cells make 184 ± 0.13% of control. These figures were not significantly different ($p>0.50$).

The time-course results for SNC80-mediated adenylyl cyclase superactivation verified our earlier observation that 4 hours with the agonist were sufficient to observe the cAMP overshoot (**Figure 17**). In this experiment, the half-life for the superactivation was 1.90 ± 0.75 hours (n=2) and the E_{max} was 216 ± 22 % of maximal forskolin-stimulated IMDM-treated control (baseline of 100% removed). This E_{max} value was consistent with cAMP overshoot values measured at 4 hours on 24 separate occasions (see **Figure 13B**).

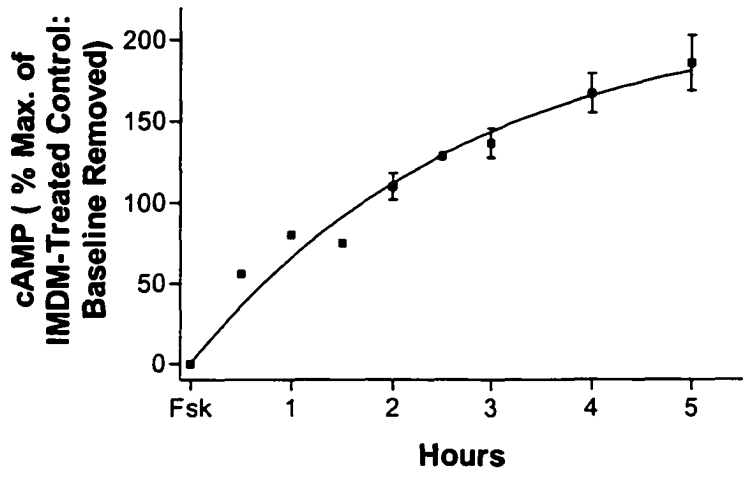

Figure 17: Time-course of adenylyl cyclase superactivation

Using a maximally-stimulating concentration of SNC80 (100 nM) for 4 hours, then washout, the 100 μM forskolin-stimulated cAMP formation was measured at various times. The SNC80-induced adenylyl cyclase superactivation had a half-life of 1.90 ± 0.75 hours and a maximal value of 216 ± 22 % of maximal forskolin-stimulated IMDM-treated control (baseline of 100% removed).

SNC80 exhibited high potency in superactivating adenylyl cyclase (**Figure 18**). In the adenylyl cyclase superactivation assay, SNC80 demonstrated an EC_{50} of 1.30 ± 1.7 nM and an E_{max} of 311 ± 20% of maximally forskolin-stimulated IMDM control. The EC_{50} of SNC80 in the adenylyl cyclase superactivation assay was

about 30-fold more potent than SNC80-mediated PLCβ stimulation and was the same as SNC80's potency in adenylyl cyclase inhibition.

Additionally, we demonstrated adenylyl cyclase superactivation induced by two other δ-opioid agonists, D-pen^2-D-pen^5-enkephalin (DPDPE) and D-ala^2-deltorphin II (**Figure 19**). The cAMP overshoot produced by DPDPE or Deltorphin II appeared to be greater than that caused by SNC80. DPDPE generated an overshoot of 399% of IMDM-treated cells using two fifteen minute washes (n=1), whereas deltorphin created 622 ± 22 % of IMDM-treated cells using two five minute washes (n=6). In comparison, SNC80 generated a cAMP overshoot of 376 ± 12% of

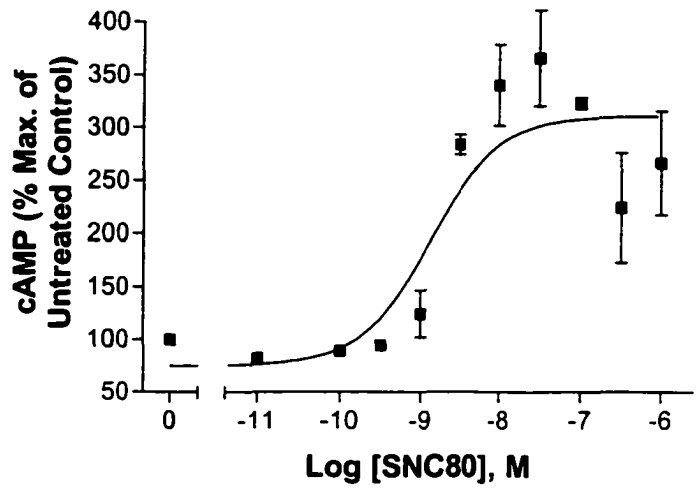

Figure 18: The potency of SNC80 in causing adenylyl cyclase superactivation

Various concentrations of SNC80 were used to pretreat hDOR/CHO cells for 4 hours, and then washed out. The E_{max} of the regression analysis was 311 ± 20% of maximally forskolin-stimulated IMDM control, (however, 30 nM SNC80 induced 366 ± 45%). The EC_{50} of SNC80 in this assay was 1.30 ± 1.7 nM (n = 2)

	CHO	B82
ACVI	+	-
ACVII	+	-
ACIX	-	+

Table 2: Adenylyl cyclase Isoforms in Chinese hamster ovary and Mouse Fibroblast B82 Cells

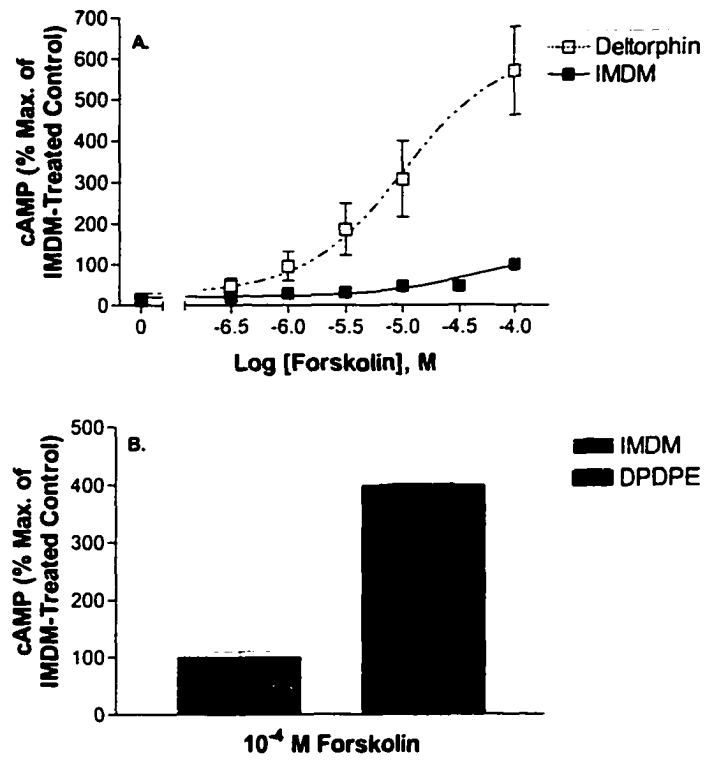

Figure 19: Adenylyl cyclase superactivation mediated by deltorphin and DPDPE

A. hDOR/CHO cells pretreated with 1 μM deltorphin, then washed exhibited a cAMP overshoot of 622 ± 22 % of IMDM-treated cells (n = 6). **B.** 500 nM DPDPE induced a cAMP overshoot of 399% (n = 1).

IMDM-treated cells using three fifteen minute washes (n=24).

Finally, we determined which adenylyl cyclase isoforms were expressed in CHO cells. Using reverse transcriptase PCR, we found clones containing transcripts that encoded for adenylyl cyclases VI and VII (**Table 2**).

DISCUSSION

Demonstration of adenylyl cyclase superactivation in hDOR/CHO cells was fortuitous, as this characteristic of chronic opioid receptor activation commonly occurs in endogenously expressing opioid systems, but is not always demonstrable in heterologous expression systems. It seems that the cellular environment must be appropriate for the

cells to be able to compensate for long-term opioid treatment in this way. As expression systems go, however, CHO cells stably expressing the human δ-opioid receptor mimic natural physiology by displaying adenylyl cyclase superactivation (adenylyl cyclase superactivation is demonstrable in the central nervous system and gut of animals treated chronically with opioids). In this assay, the method of data representation became important for allowing us to combine multiple experiments. We found that normalizing to maximally forskolin-stimulated IMDM-treated controls provided consistency from day to day, removing differences caused by daily cell number and cell behavior variation. This is likely because these intermediate raw cAMP concentrations fall within the portion of the cAMP assay standard curve where the slope is about unity. Normalizing to basal cAMP formation was not acceptable. Basal cAMP formation values tended to be small and highly variable (due to their positions on the standard curve where the slope is very shallow).

The fact that adenylyl cyclase superactivation is pertussis toxin- and naltrindole-sensitive verifies its occurrence through the human δ-opioid receptor and $G_{i/o}$-proteins, not $G_{\alpha s}$-proteins. Pertussis toxin sensitivity was important to demonstrate because the increase in adenylyl cyclase activity during adenylyl cyclase superactivation could have been easily explained by a switching of human δ-opioid receptor coupling from $G_{\alpha i/o}$ to $G_{\alpha s}$, as seen in β-adrenergic G-protein-coupled receptor systems (where receptors can switch from $G_{\alpha s}$ to $G_{\alpha i/o}$). Additionally, by blocking with naltrindole and ensuring that activation of the human δ-opioid receptor was required, we ruled out the possibility of

SNC80 acting at unknown sites in the hDOR/CHO cells to mediate adenylyl cyclase superactivation.

The 10 μM cycloheximide concentration that was chosen was shown by the Vogel laboratory at the Weizmann Institute in Israel to effectively inhibit novel protein synthesis as measured by lack of [^{3}H]-thymidine incorporation (Avidor-Reiss, et al., 1995). Although these controls were not repeated in our system, their system utilized the exact same cell line (CHO-K1) simply expressing a different opioid receptor (μ). Therefore, we were confident that the conditions optimized by Dr. Zvi Vogel's laboratory were applicable to our system. It should be noted, however, that we did not determine these conditions ourselves and these results should be interpreted with this in mind. Nevertheless, in support of our findings, the research community typically finds cycloheximide insensitivity of adenylyl cyclase superactivation to be more the rule than the exception. Furthermore, although five or more hours were required to demonstrate maximal cAMP overshoot, the overshoot was measurable and significant by 30 minutes (**Figure 17**). It is unlikely that novel expression of new or existing gene products occur in this relatively short time.

The time-course for SNC80-mediated adenylyl cyclase superactivation supported our observation that essentially equivalent cAMP overshoot results were noted at 4 and 24 hours. Based on the results of the experiment, the theoretical time to maximal adenylyl cyclase superactivation occurs by about 5.5 hours (three half-lives). However, the E_{max} determined by the regression (316 ± 22% with the baseline replaced) is consistent with

E_{max} values typically determined at 4 hours (376 ± 12% of IMDM-treated cells, n=24). This small difference is likely due to inter-experimental variability.

The potency of SNC80 in causing adenylyl cyclase superactivation turned out to be very high (1.30 ± 1.7 nM, **Figure 18**). Interestingly, this EC_{50} value mimicked SNC80's potency in inhibiting adenylyl cyclase, and not PLCβ stimulation. This was surprising because, as will be outlined in subsequent chapters, others and we have hypothesized adenylyl cyclase superactivation to be mediated by $G_{\beta\gamma}$. Because PLCβ stimulation is a $G_{\beta\gamma}$-mediated enzyme, we expected SNC80 to show the typical lower potency associated with IP_1 formation in the adenylyl cyclase superactivation assay. The fact that SNC80 had a potency more aligned with the $G_{\alpha i/o}$-mediated adenylyl cyclase inhibition indicates that either adenylyl cyclase superactivation is mediated by $G_{\alpha i/o}$ or that adenylyl cyclase superactivation is mediated by $G_{\beta\gamma}$, but that the $G_{\beta\gamma}$ is interacting with an intracellular site with a potency higher than $G_{\beta\gamma}$ interacts with PLCβ.

In hDOR/CHO cells, adenylyl cyclase superactivation was originally demonstrated using SNC80 due to this drug's water solubility (relative to the δ-opioid agonists available when such work was completed). I have since discovered that both deltorphin and DPDPE could induce adenylyl cyclase superactivation as well. In fact, deltorphin produces superactivation after the typical four-hour pretreatment, but with a greatly reduced washout time (two 5-minute IMDM washes as opposed to the three 15-minute wash requirement for SNC80). This is probably indicative of its increased water solubility and therefore more efficient removal from the CHO cell membranes. This characteristic may be important, as adenylyl cyclase superactivation is transient after

removal of the agonist. The faster one can assay for adenylyl cyclase activity with forskolin in the absence of any receptor activation, the greater and easier to measure the cAMP overshoot. Whereas my results indicate these agonists are more reliable because of their greater hydrophilicity, further experiments would have to be done to definitively verify the fast and efficient removal of these water-soluble ligands from hDOR/CHO cell culture. While the intrinsic activities of deltorphin and (-)TAN67 in adenylyl cyclase inhibition were not significantly different from SNC80, their potencies varied significantly (see Figure 6). Relative to SNC80's EC_{50} of 1.26 ± 0.37 nM, (-) TAN67 demonstrated an EC_{50} of 0.50 ± 0.1 nM, or about 2.5-fold more potent than SNC80 (n=2). Deltorphin showed an EC_{50} of 9.89 ± 7.5 nM, or about 8-fold less potent than SNC80 (n=3). With respect to adenylyl cyclase superactivation, however, supramaximal concentrations of all agonists were always used (100 nM for SNC80, and 1 μM for deltorphin and DPDPE), thereby equalizing their intrinsic activities at the receptor.

Extensive work was done by Nevo et al. in 1998 to determine which adenylyl cyclase isoforms were capable of superactivation by chronic inhibitory G-protein-coupled receptor activation (Nevo, et al., 1998). Using cDNAs for the various adenylyl cyclase isoforms transiently expressed in COS-7 cells, they found that adenylyl cyclases I, V, VI, and VIII demonstrated this characteristic, whereas II, IV, and VII were actually inhibited by chronic opioid agonist pretreatment. In the interest of thoroughly characterizing the cAMP overshoot in hDOR/CHO cells, we determined which adenylyl cyclase isoforms were expressed in CHO cells (Varga, et al., 1998). Using RT-PCR methodology with primers directed towards conserved regions in the adenylyl cyclase genes, we

transformed e. coli with the PCR products. We detected clones transformed with cDNA encoding for ACVI and ACVII in these cells. These findings were consistent with our observation of adenylyl cyclase superactivation in hDOR/CHO cells, as Nevo et al. (1998) had shown ACVI to be capable of superactivation. We were then able to attribute the cAMP overshoot to superactivation of ACVI in hDOR/CHO cells.

In summary, we have demonstrated adenylyl cyclase superactivation in Chinese hamster ovary cells stably expressing the human δ-opioid receptor. This phenomenon is directed through the receptor and $G_{\alpha i/o}$, but probably does not require activation of PLCβ, as the potencies for the two responses were significantly different. Further evidence of this was the total loss of receptor-mediated PLCβ activity after chronic treatment with SNC80 (see **Figure 11**). Adenylyl cyclase superactivation results from a modification of existing adenylyl cyclase molecules, as novel protein synthesis was not required for its production. And the adenylyl cyclase likely responsible for superactivation is adenylyl cyclase VI, the only isoform, of the two detected in CHO cells, that has been previously shown to superactivate. Due to its increased cAMP overshoot and more convenient wash times, we now utilize deltorphin II for inducing adenylyl cyclase superactivation.

I alluded earlier to the proposed involvement of $G_{\beta\gamma}$ in the formation of adenylyl cyclase superactivation. Once the cAMP overshoot was established and generally characterized, we undertook the task of investigating the molecular mechanism of its formation, which led us to our next hypothesis.

CHAPTER 4: THE INVOLVEMENT OF $G_{\beta\gamma}$ SUBUNITS IN ADENYLYL CYCLASE SUPERACTIVATION

INTRODUCTION

Because adenylyl cyclase superactivation in Chinese hamster ovary cells stably expressing the human δ-opioid receptor (hDOR/CHO cells) was pertussis toxin-sensitive, we were confident that $G_{i/o}$ G-proteins were required. After G-protein activation, the signal transduction diverges into $G_{\alpha i/o}$- and $G_{\beta\gamma}$-mediated events. As stated earlier, $G_{\alpha i/o}$ subunits were responsible for acute inhibition of adenylyl cyclase by interacting directly with available adenylyl cyclase isoforms, which we have shown to be ACVI and ACVII in Chinese hamster ovary cells (**Table 2**). We were confident that the adenylyl cyclase isoform in CHO cells responsible for causing adenylyl cyclase superactivation was ACVI. This was partially because, of these two types, only ACVI has been shown to superactivate and ACVII seems to be stimulated by acute opioids in both endogenously expressing tissues, such as the gut and transfected COS-7 cells (Wang, et al., 1996; Nevo, et al., 1998). Additionally, we know which $G_{\alpha i/o}$ subunits are expressed in these cells (**Table 1**).

The majority of signals mediated by G-protein-coupled receptors appear to be through $G_{\beta\gamma}$. We therefore hypothesized that $G_{\beta\gamma}$ subunits were more likely mediators of the compensatory adenylyl cyclase superactivation. This is in part due to the diversity of signals that $G_{\beta\gamma}$ subunits are known to transduce, such as phospholipase A_2 (PLA_2), $PLC\beta_{2,3}$, and PLD activation, subsequent protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (PI-3K) activation (as well as

potassium channel opening and voltage-dependent calcium channel inhibition in excitable tissues). We figured that one or more of these effectors could lead to the persistent increase in adenylyl cyclase activity that appears to counteract the $G_{\alpha i/o}$-mediated inhibition.

To investigate the involvement of $G_{\beta\gamma}$, we attempted three different approaches. The first relied upon transfection of antisense DNA directed toward a conserved region in the G_β subunits. To date, five G_β subunits have been cloned and all share about 67% homology with each other, and about 90% between the most common subtypes, β_{1-4}. Alternatively, thirteen different members, sharing as little as 22% homology, comprise the G_γ subunits. Therefore, our goal was to use this antisense approach to knock down the expression of G_β in CHO cells stably expressing the human δ-opioid receptor. Because signaling through $G_{\beta\gamma}$ requires G_β and G_γ subunits to be associated, reducing the available G_β protein would effectively prevent signaling through the $G_{\beta\gamma}$ complex.

The second two methods for deterring signaling through $G_{\beta\gamma}$ were to introduce different scavengers of $G_{\beta\gamma}$ subunits. This approach relies on the ability of the scavenger to have a high enough concentration and affinity for $G_{\beta\gamma}$ to associate with and tie up free $G_{\beta\gamma}$ subunits as they are released from activated $G_{\alpha i/o}$ (**Figure 20**). The first scavenger, phosducin, is a common protein whose physiological role is thought to be that of a naturally occurring $G_{\beta\gamma}$ scavenger (Schulz, 2001). The rat phosducin construct arrived from the American type culture collection (ATCC, Manassas, VA) in the pBluescript vector, which was not suitable for mammalian expression. We subcloned the phosducin

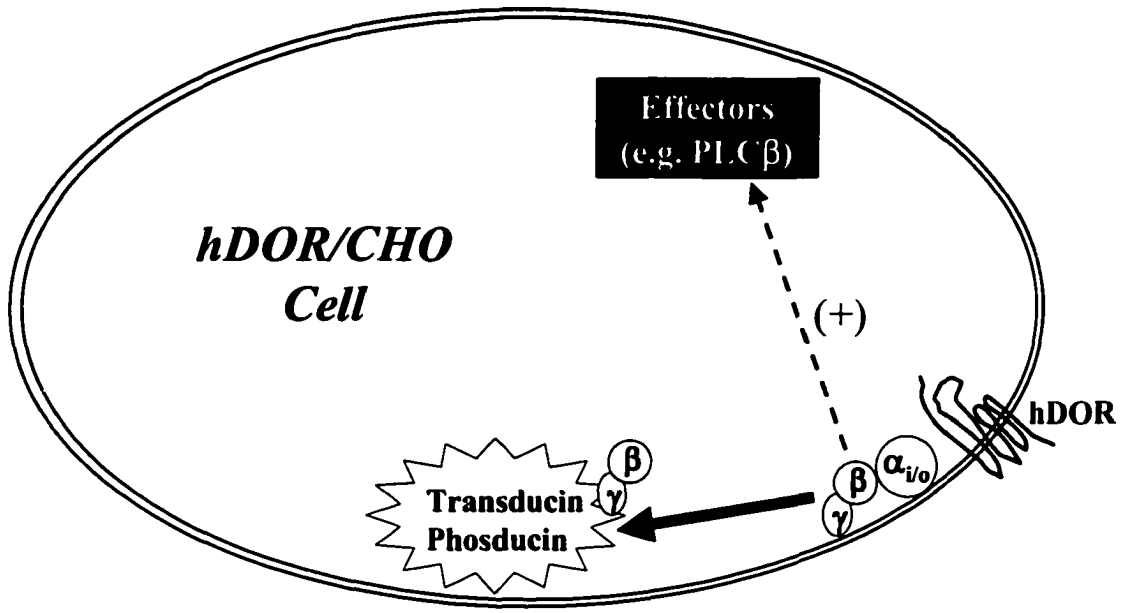

Figure 20: How transducin and phosducin scavenge $G_{\beta\gamma}$

When liberated from their cognate $G_{\alpha i/o}$ subunits, $G_{\beta\gamma}$ typically interact with effectors to transduce G-protein-coupled receptor-mediated signals. These free $G_{\beta\gamma}$ subunits can be intercepted by an abundance of GDP-bound $G_{\alpha t}$ subunits or by phosducin before they get the chance to find their usual effectors.

gene into pcDNA 3.1, which codes for G418 (neomycin) resistance. This was important because the human δ-opioid receptor gene is in pcDNA 3.1 encoding for hygromycin resistance, and our aim was to create transient co-transfectants. By growing the co-transfected cells in both hygromycin and G418, we would be able to select for clones expressing both constructs.

The next scavenger we used was the G_{α} subunit of the retinal G-protein, transducin ($G_{\alpha t}$). In our earlier examination of the pertussis toxin-sensitive G-protein α subunit expression, we detected $G_{\alpha t}$ in CHO cells. This was a bit surprising because, not

only are the Chinese hamster ovary cells not of retinal origin, but they are not even a nervous or excitable tissue. Others in the laboratory were interested in examining the coupling of $G_{\alpha t}$ to the human δ-opioid receptor in CHO cells, and created a pertussis toxin-insensitive mutant of $G_{\alpha t}$ to examine this. This construct was stably expressed in hDOR/CHO cells, relying on the double negative selection as outlined above. We discovered that this cell line could be employed to examine the effects of $G_{\beta\gamma}$ scavenging on adenylyl cyclase superactivation (as well as other responses). Here, the theory is that overexpression of the GDP-bound $G_{\alpha t}$ subunit would serve as a sponge to absorb any free $G_{\beta\gamma}$. This approach relies on the inability of the receptor of interest, here the human δ-opioid receptor, to significantly couple to and activate the overexpressed $G_{\alpha t}$, leaving inactive, GDP-bound $G_{\alpha t}$ to scavenge $G_{\beta\gamma}$. Many laboratories have used $G_{\alpha t}$ as a $G_{\beta\gamma}$ scavenger in a variety of systems, including CHO cells (Federman, et al., 1992; Avidor-Reiss, et al., 1996; Yoshimura, et al., 1996; Selbie, et al., 1997; Olianas and Onali, 1999). One advantage of our system was the pertussis toxin-insensitive nature of the α-transducin. This allowed us to examine any coupling of $G_{\alpha t}$ to adenylyl cyclase as a control for the possibility that $G_{\alpha t}$ simply inhibits the association of endogenous G-proteins with the receptor by directly interacting with the human δ-opioid receptor. Normally, $G_{\alpha t}$ serves to activate phosphodiesterases, leading to a decrease in cAMP formation (much like what is seen by $G_{\alpha i/o}$ activation and adenylyl cyclase inhibition). Therefore, $G_{\alpha t}$ would be considered to be working well as a scavenger if it interfered with $G_{\beta\gamma}$ and not with receptor-mediated endogenous G-protein activation.

As mentioned previously, $G_{\beta\gamma}$ has been shown to stimulate PLCβ_2 and PLCβ_3 enzymes. Interestingly, Dickenson and Hill (1998) demonstrated expression of PLCβ_3 (while looking for PLCβ_{1-4}) in CHO cells, and we have shown PLCβ activation by the human δ-opioid receptor in CHO cells that is pertussis toxin-sensitive. These results indicate that, because $G_{\alpha i/o}$ subunits are not known to activate PLCβ, this signal is being directed through $G_{\beta\gamma}$. Therefore, we were able to use this measurement as a positive control for $G_{\beta\gamma}$ scavenge. We would expect an attenuation of PLCβ activation (and subsequent IP_1 formation) when $G_{\beta\gamma}$ signaling is interrupted. We have used this positive control when examining any of the described techniques for interrupting signaling through $G_{\beta\gamma}$.

METHODS

Creation of hDOR/$G_{\alpha t}$/CHO cells and cell culture

Chinese hamster ovary cells stably expressing the human δ-opioid receptor (hDOR/CHO) (Malatynska, et al., 1995) were transfected with a pertussis toxin-insensitive mutant of the α-subunit of transducin-1 (hDOR/$G_{\alpha t}$/CHO) as previously described (Varga, et al., 2000). Both hDOR/CHO and hDOR/$G_{\alpha t}$/CHO cells were plated in 24-well polystyrene plates (Costar, Cambridge, MA) at a density of 65,000 cells per well and grown in a humidified incubator at 37° C (5% CO_2) for 48 hours in Ham's F-12 medium (Invitrogen, Carlsbad, CA) with 10% fetal calf serum prior to assay. The expression of $G_{\alpha t}$ was verified by Western blotting (Varga, et al., 2000).

Treatment of hDOR/CHO cells with G_β antisense

Antisense treatment conditions, such as oligonucleotide DNA to DOSPER® concentration ratios, were determined by referring to the literature included with the DOSPER® lipid transfection reagent (Boehringer Mannheim). The ratios used are indicated in **Figure 21**. Two treatment times were used: one 6-hour antisense transfection in serum-starved cells followed by an 18-hour recovery in Ham's F-12 growth media with fetal bovine serum and two 6-hour antisense transfections in serum-starved cells over a 48-hour period, each transfection followed by an 18-hour recovery in Ham's F-12 growth media. Following this treatment, cells were assayed for SNC80-mediated IP_1 formation as described in the GENERAL METHODS (Chapter 2) The oligonucleotide sequence was: 5'-G*T*C*AAA GAG GCG GCA TGT GGC ATC*A*T*C-3', where asterisks refer to phosphorothioate bonds.

Transient Transfection of CHO Cells with phosducin and the human δ-opioid receptor

CHO cells were plated in 100 mm polystyrene plated plates (Costar) and grown until 50% confluent. Using 4 μg each of phosducin and human δ-opioid receptor cDNA or 4 μg of human δ-opioid receptor cDNA alone, cells were transfected using the dextran/chloroquine method. Cells were subsequently plated and grown in Ham's F-12 media supplemented with 400 μg/ml G418 (neomycin) and 400 μg/ml hygromycin to select for cells expressing both the human δ-opioid receptor and phosducin (hDOR/phosducin/CHO) or 400 μg hygromycin alone for control cells (hDOR/CHO).

Following transfection, hDOR/CHO and hDOR/phosducin/CHO cells were plated at 65,000 cells per well in 24-well plates and assayed for adenylyl cyclase or PLCβ activity as described in the GENERAL METHODS.

RESULTS

The first attempt to block $G_{\beta\gamma}$-mediated signaling utilized antisense directed towards $G_{\beta 1-4}$. Using DOSPER®, a cationic lipid antisense transfection system, which the manufacturer assured worked for oligonucleotide transfection of Chinese hamster ovary cells, I was unable to demonstrate significant G_{β} knockdown. The effectiveness of the treatment was measured by human δ-opioid receptor-mediated PLCβ activation and subsequent IP_1 formation, a $G_{\beta\gamma}$-mediated effect (**Figure 21**). In this figure, a series of conditions were created by varying the relative

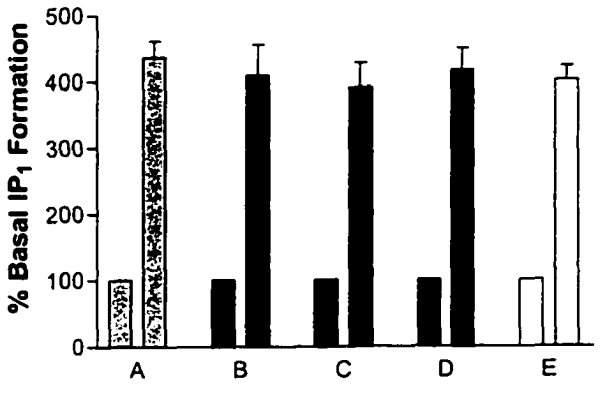

Figure 21: SNC80-mediated IP_1 formation after antisense pretreatment

hDOR/CHO cells were treated with various ratios of DOSPER® cationic lipid to antisense oligonucleotide concentration. **A.** 10 μg DNA:3 μg lipid, **B.** 10 μg DNA:12 μg lipid, **C.** 20 μg DNA:3 μg lipid, **D.** 20 μg DNA:12 μg lipid, **E.** 0 μg DNA:0 μg lipid. Cells were treated with antisense for two 6-hour periods (over 48 hours). There was no significant difference between any of the treatment groups and the control group (**E.**) (n = 3).

concentrations of DOSPER lipid to DNA according to the manufacturer's suggestions. Shown are the basal and 1 μM SNC80-stimulated (maximally-stimulated) values, represented as a percent of basal. Basal values for each experiment were 284 ± 10, 207 ± 23, and 166 ± 6 CPM (average ± SEM). In spite of the different conditions, there was no significant difference between any of the cells' ability to stimulate PLCβ.

Because of the difficulties presented by antisense methodology, we decided to

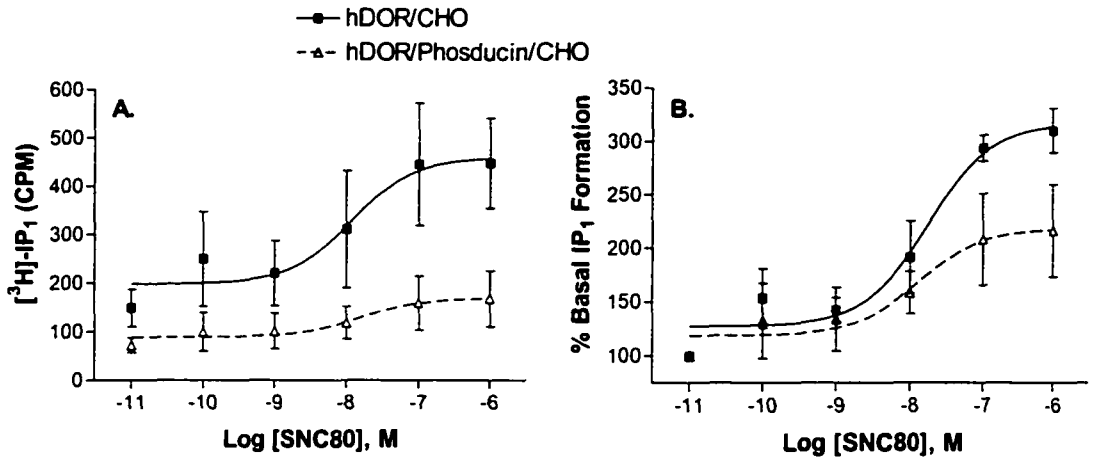

Figure 22: SNC80-Mediated PLCβ Stimulation in hDOR/phosducin/CHO cells

A. CHO cells transiently transfected with the human δ-opioid receptor showed an IP_1 formation E_{max} value of 460 ± 35 CPM and a basal activity of 197 ± 26 CPM. CHO cells transiently transfected with the human δ-opioid receptor and phosducin displayed an E_{max} of 169 ± 11 CPM and a basal activity of 88 ± 7 CPM. The EC_{50} values for singly or co-transfected CHO cells were 12 ± 20 nM and 15 ± 20 nM, respectively. **B.** The same data converted to a percent of basal IP_1 formation clearly demonstrates the effect of phosducin on δ-opioid receptor-mediated PLCβ stimulation. Human δ-opioid receptor-transfected cells exhibited an E_{max} of 316 ± 20% of basal, whereas co-transfected cells showed a significantly reduced E_{max} of 218 ± 12% of basal (n = 3).

transiently transfect phosducin, nature's $G_{\beta\gamma}$ scavenger, along with the human δ-opioid receptor into CHO cells. Utilizing the dextran/chloroquine method for transfection, these transfected cells were subsequently plated and examined for the ability of SNC80 to stimulate PLCβ, as well as superactivate adenylyl cyclase. In **Figure 22**, co-transfected cells display a diminished maximal SNC80-mediated IP_1 formation of 218 ± 12% of basal, whereas cells transfected with the human δ-opioid receptor alone demonstrated a value of 316 ± 20% of basal. These values were significantly different ($p<0.01$, n=3).

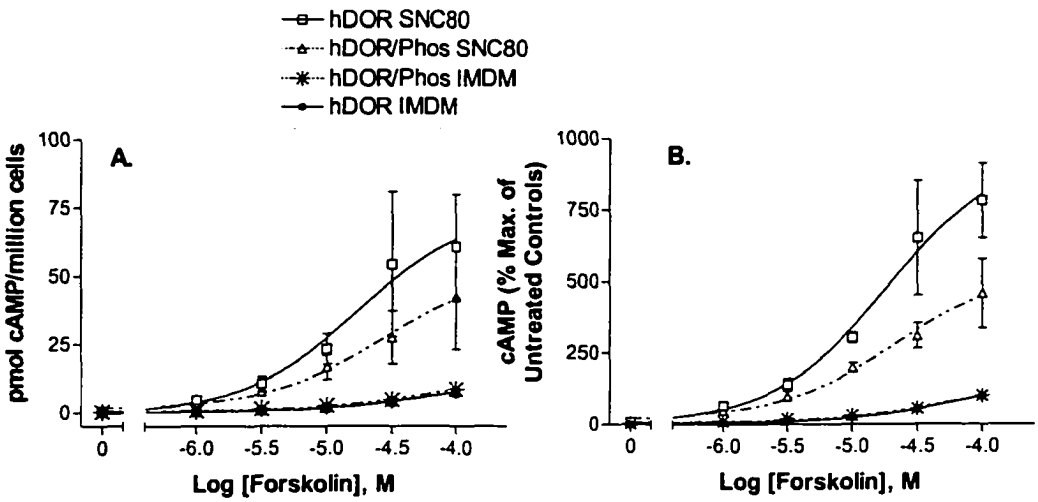

Figure 23: SNC80-mediated superactivation in hDOR/phosducin/CHO cells

A. CHO cells transiently transfected with the human δ-opioid receptor showed a cAMP overshoot of 61.5 ± 8 pmol cAMP per million cells. CHO cells transiently transfected with the human δ-opioid receptor and phosducin displayed a significantly diminished cAMP overshoot of 39 ± 5 pmol per million cells ($p<0.05$, n = 3). **B.** As a percent of maximally stimulated iMDM-treated control, hDOR/CHO cells demonstrated a cAMP overshoot of 947 ± 65% of control. Phosducin expression significantly reduced this value to 540 ± 35% of control ($p<0.01$, n = 3).

Neither the bottom of the curve nor the EC_{50} values were statistically different between the regression analyses. The basal IP_1 formation values were 197 ± 26 and 88 ± 7 CPM for hDOR/CHO and hDOR/phosducin/CHO cells, respectively, and were significantly different ($p<0.01$).

In **Figure 23**, adenylyl cyclase superactivation was significantly attenuated by transient transfection of phosducin. Whereas untransfected cells demonstrated an SNC80-mediated cAMP overshoot of 947 ± 65% of control, phosducin expression significantly reduced this value to 540 ± 35% of control ($p<0.01$). None of the other values in the regression analysis was significantly different between the two groups. The raw maximal IMDM-treated values were 12.8 ± 0.3 and 13.1 ± 1 pmol cAMP/million cells for hDOR/CHO and hDOR/phosducin/CHO cells, respectively, without any significant difference between these groups.

In addition to phosducin, we used a pertussis toxin-insensitive mutant of α-transducin-1 ($G_{\alpha t}$), another $G_{\beta\gamma}$ scavenger, which also happened to be paradoxically endogenously expressed in Chinese hamster ovary cells. Stable human δ-opioid receptor- and $G_{\alpha t}$-expressing Chinese hamster ovary cells (hDOR/$G_{\alpha t}$/CHO) were created and characterized by [^{3}H]-naltrindole saturation binding and their SNC80-mediated inhibition of forskolin-stimulated adenylyl cyclase in the absence and presence of pertussis toxin to examine the integrity of $G_{\alpha i/o}$-mediated signaling in the presence of transducin. SNC80-mediated PLCβ activation was assessed to verify $G_{\beta\gamma}$ scavenge. Finally, adenylyl cyclase superactivation was examined in these cells and compared with hDOR/CHO cells.

[^{3}H]-naltrindole saturation binding was accomplished at 37° C and allowed to reach equilibrium over a 3-hour period (**Figure 24**). B_{max} values for hDOR/CHO and hDOR/$G_{\alpha t}$/CHO cells were calculated to be 1831 ± 150 and 1191 ± 92 fmol of receptor per million cells, respectively. The expression of human δ-opioid receptors in hDOR/$G_{\alpha t}$/CHO cells was significantly lower ($p<0.05$, n=2). The K_d for naltrindole in hDOR/CHO cells was 836 ± 160 pM and 593 ± 120 pM in hDOR/$G_{\alpha t}$/CHO cells. These values were not significantly different ($p>0.50$, n=2).

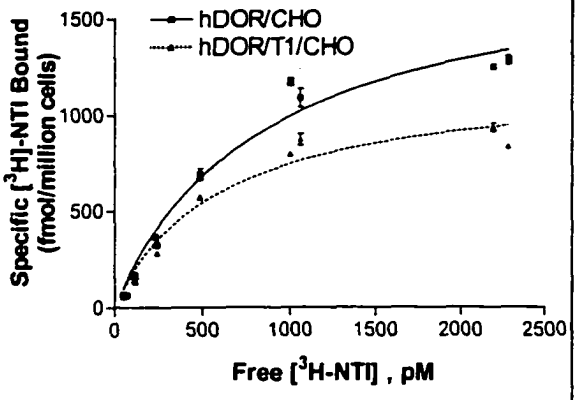

Figure 24: [^{3}H]-naltrindole binding in hDOR/$G_{\alpha t}$/CHO cells

The density of human δ-opioid receptors in hDOR/CHO cells (B_{max}) was 1831 ± 150 fmol per million cells. The B_{max} in hDOR/$G_{\alpha t}$/CHO cells was significantly reduced to 1191 ± 92 fmol per million cells. The K_d values for naltrindole in these cells were not significantly different from one another.

Inhibition of 0.1 mM forskolin-stimulated adenylyl cyclase by SNC80 was unaffected by $G_{\alpha t}$ expression (**Figure 25**). In hDOR/CHO and hDOR/$G_{\alpha t}$/CHO cells, SNC80 inhibited forskolin-stimulated adenylyl cyclase by 98 ± 11% and 97 ± 15%, respectively, with EC_{50} values of 1.6 ± 1 nM and 1.6 ± 2 nM, respectively. There was no statistically significant difference in any of the regression parameters between the two clones.

Pertussis toxin pretreatment of hDOR/$G_{\alpha t}$/CHO cells completely abolished human δ-opioid receptor-mediated adenylyl cyclase inhibition (**Figure 26**) as it does in hDOR/CHO cells. Whereas untreated cells exhibited an E_{max} of 15.6 ± 8% of control with an EC_{50} of 4.9 ± 7 nM, pertussis toxin pretreated cells showed no measurable inhibition.

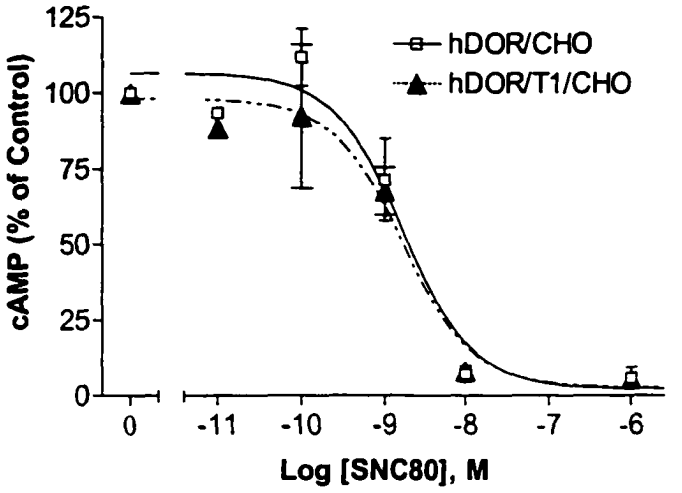

Figure 25: SNC80-inhibition of adenylyl cyclase in hDOR/$G_{\alpha t}$/CHO cells

SNC80 demonstrated an inhibition of 100 μM forskolin-stimulated cAMP formation of 98 ± 11 % of control in hDOR/CHO cells and 97 ± 15% of control in hDOR/$G_{\alpha t}$/CHO cells. The EC_{50} of SNC80 in hDOR/CHO cells was 1.6 ±1 nM and 1.6 ± 2 nM in hDOR/$G_{\alpha t}$/CHO cells. There was no significant difference in any of the regression values ($p>0.50$, $n = 3$).

In **Figure 27**, $G_{\alpha t}$ expression significantly attenuated SNC80-mediated maximal IP_1 formation from 361 ± 17% to 206 ± 9% ($p<0.01$). The EC_{50} values were not significantly different (41 ± 40 nM in hDOR/CHO cells and 9.8 ± 10 nM in hDOR/$G_{\alpha t}$/CHO cells). Basal IP_1 formation in hDOR/CHO and hDOR/$G_{\alpha t}$/CHO cells was 589 ± 460 and 1012 ± 90 CPM, respectively. These values were not statistically different from one another.

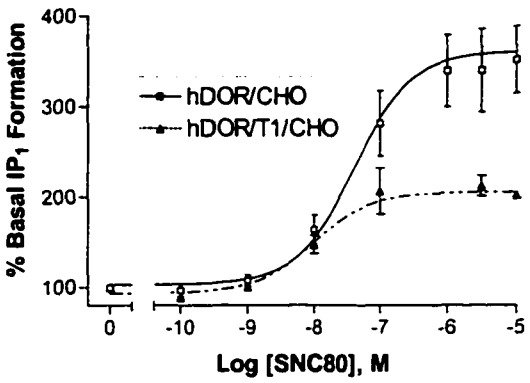

Figure 27: SNC80-mediated IP_1 formation in hDOR/$G_{\alpha t}$/CHO cells
Whereas SNC80 maximally stimulated PLCβ by 361 ± 17% in hDOR/CHO cells, the maximal stimulation in hDOR/$G_{\alpha t}$/CHO cells was significantly reduced to 206 ± 9% ($p<0.01$, n=3). There was no difference in their EC_{50} values. (Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.)

values for IMDM- and SNC80-treated hDOR/$G_{\alpha t}$/CHO cells (cAMP overshoot) was not significant, indicating complete abolishment of adenylyl cyclase superactivation.

Adenylyl cyclase superactivation was significantly attenuated by expression of $G_{\alpha t}$ (**Figure 28**) ($p<0.01$). Whereas hDOR/CHO cells exhibited forskolin E_{max} values of 164 ± 8% and 376 ± 12% in IMDM- and 100 nM SNC80-treated cells, respectively, hDOR/$G_{\alpha t}$/CHO cells revealed 166 ± 14% and 163 ± 8%. The difference between E_{max}

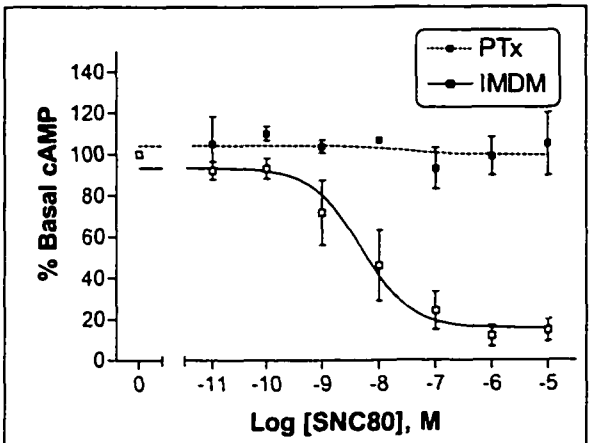

Figure 26: Pertussis toxin sensitivity of SNC80-inhibition in hDOR/$G_{\alpha t}$/CHO cells
In IMDM-treated cells, SNC80 inhibited 100 μM forskolin-stimulated cAMP formation by 84 ± 8% of control with an EC_{50} of 4.9 ± 7 nM. 18 hour with 50 ng/ml pertussis toxin completely abolished this response. (n = 5).

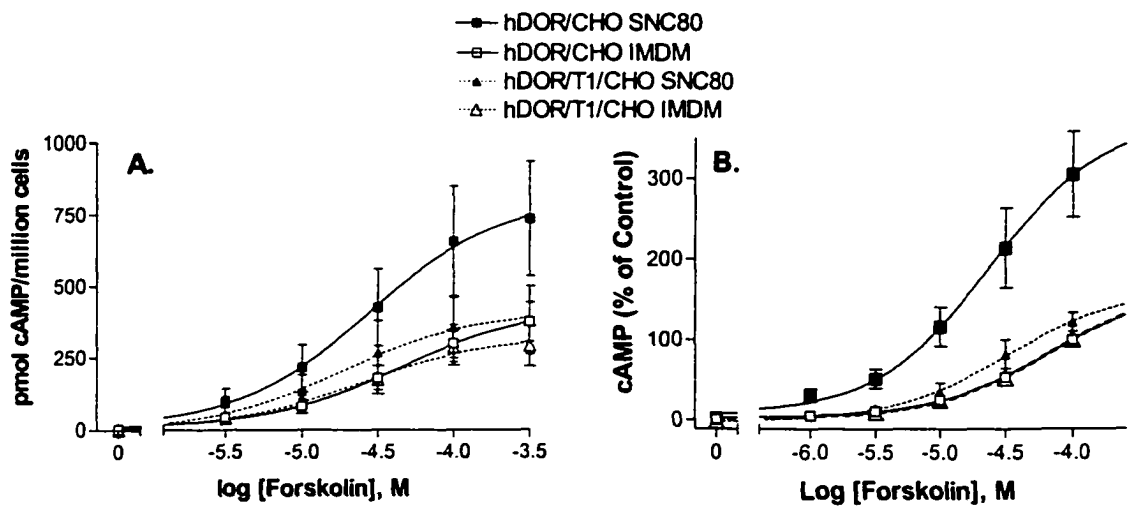

Figure 28: Adenylyl cyclase superactivation in hDOR/$G_{\alpha t}$/CHO cells

A. 100 nM SNC80 induced a cAMP overshoot of 382 ± 27 pmol per million cells in hDOR/CHO cells ($p<0.01$, n = 5). The same treatment in hDOR/$G_{\alpha t}$/CHO cells resulted in a significantly reduced cAMP overshoot of 86 ± 31 pmol per million cells ($p<0.01$, n = 3). **B.** As a percent of maximally stimulated IMDM-treated control, hDOR/CHO cells exhibited a 100 nM SNC80-induced cAMP overshoot of 376 ± 12%, whereas $G_{\alpha t}$ expression significantly reduced this value to 163 ± 8% ($p<0.01$, n = 4). The cAMP overshoot exhibited by hDOR/$G_{\alpha t}$/CHO cells was not statistically significant ($p>0.50$).

DISCUSSION

We initially decided to employ DNA antisense methodology due to its high degree of specificity and the potentially expeditious results. We had not anticipated the difficulties in optimizing the conditions that were necessary for knockdown to occur. To aid in transfection efficiency, we used the cationic lipid, DOSPER®, although it did not

appear to have any effect. We did not measure the efficiencies of the transfections because we had at our disposal an established functional positive control. As outlined above, PLCβ_3 activation by the human δ-opioid receptor was shown to be mediated by $G_{\beta\gamma}$ subunits in other laboratories. Evidence of loss of G_β (and subsequent $G_{\beta\gamma}$ signaling) would have been demonstrable by a reduction in maximal IP_1 formation (or by a rightward shift in the SNC80 concentration-response curve if spare $G_{\beta\gamma}$ subunits were a factor). The basal values fluctuated insignificantly, and, as noted in **Figure 21**, there was no change in the percent basal maximal IP_1 formation after 48 hr antisense DNA pretreatment. **Figure 21** comprises three experiments, however, a number of attempts at G_β knockdown utilizing different DNA and DOSPER concentrations, treatment times, and multiple antisense DNA in as much as a 72 hour period were examined. None of these modifications had any affect on SNC80-mediated IP_1 formation (data not shown). For these reasons, we shifted our approach by attempting to selectively scavenge free $G_{\beta\gamma}$.

There have been a number of methods dedicated to scavenging $G_{\beta\gamma}$. We initially opted for phosducin because it has been characterized as a natural $G_{\beta\gamma}$ scavenger. Phosducin has been successfully used in this capacity on many occasions, both transiently and stably expressed. As $G_{\beta\gamma}$ scavengers go, phosducin was considered because it was the least likely to interfere with cellular processes other than those mediated by $G_{\beta\gamma}$, such as opioid ligand affinity and $G_{\alpha i/o}$-mediated downstream effects, and even intracellular homeostasis unrelated to the human δ-opioid receptor.

When I transiently transfected phosducin into hDOR/CHO cells, and measured SNC80-mediated IP_1 formation 48 hr post-transfection, there appeared to be an indication

of $G_{\beta\gamma}$ scavenging, but I could not establish statistical significance (data not shown). A heterogeneous population of transfected and untransfected cells grown in media lacking antibiotic selection likely confounded these initial experiments. As noted in **Figure 22**, however, significant $G_{\beta\gamma}$ scavenge by phosducin was accomplished by growing the freshly co-transfected hDOR/phosducin/CHO cells in media containing antibiotic selection for both the hDOR and phosducin constructs. The result was a significant 31 ± 17% of basal reduction in maximal IP_1 formation in phosducin-transfected cells. The only deficiency was that human δ-opioid receptor densities were not measured by saturation binding in these cells. The small, but significant reduction in the basal IP_1 formation value for hDOR/phosducin/CHO cells (109 ± 66 CPM reduction, n=3) might have indicated a reduction in human δ-opioid receptor expression. However, this result could also be explained as an inhibition of constitutive receptor activation or an inhibition of receptor-independent spontaneous guanine nucleotide exchange (and subsequent $G_{\beta\gamma}$ liberation) and the resultant PLCβ activation. Because the data were transformed as a percent of each condition's basal, these factors should have been accounted for. In fact, since the basal IP_1 formation in hDOR/phosducin/CHO cells is lower, one might have expected an unusually large percent of basal, which was not the case. The overall activation of PLCβ (top minus bottom) in the two transfection conditions was 263 ± 124 CPM for hDOR-transfected cells and 81 ± 36 CPM for the hDOR and phosducin co-transfected cells, values which were significantly different ($p<0.05$, n=3). Thus, even the

overall raw IP_1 formation is significantly different between the two groups, strongly indicating that phosducin is indeed working as predicted, by scavenging free $G_{\beta\gamma}$.

Once we were convinced that the transiently transfected phosducin was scavenging $G_{\beta\gamma}$, we tested whether $G_{\beta\gamma}$ was required for adenylyl cyclase superactivation. In **Figure 23**, maximal cAMP formation was significantly attenuated in the phosducin-transfected cells. As these experiments were conducted on the same transfected cell population that was used to determine the SNC80-mediated IP_1 formation in **Figure 22**, we concluded that phosducin was equally effective in scavenging $G_{\beta\gamma}$ in these cells. Both as a percent of maximal IMDM-treated control and pmol cAMP per million cells, the attenuation of maximal forskolin-stimulated cAMP formation was statistically significant ($p<0.05$). Again, a reduced expression of the human δ-opioid receptor could have accounted for the superactivation results after phosducin transfection, but for the reasons outlined above, we do not believe that this was the case. Other than [^{3}H]-naltrindole saturation binding, human δ-opioid receptor expression could be relatively quantified by SNC80-mediated inhibition of forskolin-stimulated cAMP formation, or IP_1 formation in the presence of spare receptors. Using these techniques, the intrinsic activity (E_{max}) or EC_{50} (depending on the presence of spare receptors) of the opioid agonist would reflect the total number of functional receptors. Assuming these transiently transfected cells expressed spare human δ-opioid receptors, we would have noted a shift in the EC_{50} in **Figure 22** if different densities of these receptors were expressed. As there was no significant shift in the SNC80 concentration-response curves (17.7 ± 22 and 13.2 ± 20 nM for hDOR/CHO and hDOR/phosducin/CHO cells, respectively), it was unlikely that

the receptor densities were significantly different. The data from these IP_1 formation and adenylyl cyclase superactivation experiments implicated an important role for $G_{\beta\gamma}$ in adenylyl cyclase superactivation, but as an added measure, they were confirmed by expression of another $G_{\beta\gamma}$ scavenger, $G_{\alpha t}$ (α-transducin).

Much like phosducin, $G_{\alpha t}$ has been widely employed as a $G_{\beta\gamma}$ scavenger when overexpressed, both transiently and stably. Phosducin expression in CHO cells presents the irreconcilable dilemma of exogenous protein expression and any unaccountable effects that the introduction of a novel protein may produce. The case for phosducin has been made by the indication that its role in nature serves entirely to scavenge $G_{\beta\gamma}$ and that it has not been shown to interfere with other cellular components. However, we previously demonstrated the endogenous expression of $G_{\alpha t}$ in CHO cells, so when we chose to utilize $G_{\alpha t}$ as a $G_{\beta\gamma}$ scavenger, we averted the potential criticism involving the introduction of an exogenous protein into these cells. Because we required consistency and planned to extensively characterize and study the expression of both the human δ-opioid receptor and $G_{\alpha t}$, we opted for the creation of a stable CHO cell line expressing both of these constructs, termed hDOR/$G_{\alpha t}$/CHO cells.

Much like the hDOR/phosducin/CHO functional characterization, we determined the reduction in IP_1 formation in $G_{\alpha t}$-expressing cells (**Figure 27**). In hDOR/$G_{\alpha t}$/CHO cells, percent basal IP_1 formation was significantly reduced by 59 ± 12%, notably larger than the reduction observed in the hDOR/phosducin/CHO cells. This indicated a more substantial $G_{\beta\gamma}$ scavenge than the phosducin-expression provided. As for basal IP_1

formation (in CPM) for the two cell lines, the hDOR/CHO cells averaged 589 ± 900 (Ave. ± S.D.), whereas the hDOR/$G_{\alpha t}$/CHO cells averaged 1012 ± 200 (n=4). There was no statistically significant difference observed using the Student's t-test to compare these values. However, the transformation applied in the IP_1 formation results significantly decreased the day-to-day variability between experiments and allowed for simpler determination of statistical significance between the control and treatment groups.

We measured [^{3}H]-naltrindole saturation binding to determine the total expression of human δ-opioid receptors in hDOR/$G_{\alpha t}$/CHO cells (**Figure 24**). Interestingly, the hDOR/$G_{\alpha t}$/CHO cells had a significantly lower B_{max} value. Preliminary experiments (n=2) using whole cell binding showed that the B_{max} value for [^{3}H]-naltrindole saturation binding was 1831 ± 150 fmol/million cells for hDOR/CHO cells and 1191 ± 92 fmol/million cells. Initially, this concerned us because it might have accounted for the reduction in SNC80-mediated maximal IP_1 formation in these cells. But upon further investigation, we did not believe the difference in human δ-opioid receptor B_{max} values to influence PLCβ activation and adenylyl cyclase superactivation.

Although we determined a difference in receptor expression, the SNC80-mediated IP_1 formation shown in **Figure 27** indicates no statistically significant shift in EC_{50} values. Whereas hDOR/CHO cells exhibited an SNC80 EC_{50} of 41 ± 40 nM, hDOR/$G_{\alpha t}$/CHO cells showed 9.8 ± 10 nM ($p>0.05$). If anything, there appeared to be a trend toward greater potency in the cells with fewer receptors. Assuming the presence of spare receptors, a decrease in the potency of SNC80 would have been noted. These experiments indicated that either α-transducin scavenged $G_{\beta\gamma}$ as predicted, or that there

was no receptor reserve and the modest loss of receptor density in hDOR/$G_{\alpha t}$/CHO cells reduced SNC80's intrinsic activity.

We also examined SNC80-mediated inhibition of forskolin-stimulated cAMP formation. While there was a slight increase in the raw untreated adenylyl cyclase activity data (pmol cAMP per million cells, maximally forskolin-stimulated) for hDOR/$G_{\alpha t}$/CHO cells, the two curves just about overlapped when represented as a percent of this activity. **Figure 25** demonstrated no shift in either E_{max} or EC_{50}. If there were no spare receptors, a decrease in the E_{max} would have been observed, much like the decrease seen in **Figure 27**. If spare receptors were present, as they would likely be in this overexpressed system, a shift in the EC_{50} would have been noted. The fact that these curves were identical indicated that the small difference in B_{max} values shown in **Figure 24** translated into a functional insignificance.

To further bolster our confidence in the data, we examined stimulation of [^{35}S]-GTPγS binding in these cells. We previously published G-protein activation data using SNC80-mediated [^{35}S]-GTPγS binding that shows no significant difference in E_{max} between the cell lines (231 ± 19% for the hDOR/CHO cells and 193 ± 36% for the hDOR/$G_{\alpha t}$/CHO cells (Varga, et al., 2000). Because we demonstrated some coupling of the human δ-opioid receptor to $G_{\alpha t}$, the E_{max} in hDOR/$G_{\alpha t}$/CHO cells was acquired by determining the difference between naïve and pertussis toxin-treated cells. Since the $G_{\alpha t}$ was pertussis toxin-insensitive, this difference allowed us to ascertain the amount of human δ-opioid receptor-mediated stimulation of native G-proteins. Equal stimulation of

[^{35}S]-GTPγS binding to native G-proteins in the two cell lines indicated that equal amounts of $G_{\alpha i/o}$ were activated and that equal quantities of $G_{\beta\gamma}$ were released.

One other control we conducted was to pretreat hDOR/$G_{\alpha t}$/CHO cells with pertussis toxin, eliminating the possibility of activating endogenous $G_{\alpha i/o}$ G-proteins, while preserving the potential activation of the pertussis toxin-insensitive $G_{\alpha t}$. Using this technique, we would be able to detect any coupling of the human δ-opioid receptor to α-transducin because α-transducin couples to both adenylyl cyclase inhibition and activation of cAMP phosphodiesterases, both resulting in a reduction in cAMP formation. As seen in **Figure 26**, pertussis toxin pretreatment completely eliminated SNC80-mediated inhibition of forskolin-stimulated cAMP formation. This control was important for determining that the mechanism of $G_{\alpha t}$'s action was indeed $G_{\beta\gamma}$ scavenge, and not inhibition of endogenous G-protein activation by simple competition.

We were confident that α-transducin was scavenging $G_{\beta\gamma}$ without causing widespread non-specific effects related to human δ-opioid receptor activation and downstream $G_{\alpha i/o}$-mediated events. Convinced of this, we measured adenylyl cyclase superactivation in these cells. As seen in **Figure 28**, the SNC80-pretreated maximal forskolin-stimulated cAMP formation in hDOR/$G_{\alpha t}$/CHO cells was significantly lower than in control cells ($p<0.01$) and, in fact, demonstrated no statistically significant overshoot. This finding strongly implicated a vital role for $G_{\beta\gamma}$ subunits in the formation of opioid agonist-mediated superactivated adenylyl cyclase.

To summarize, $G_{\alpha i/o}$ subunits, activated in response to human δ-opioid receptor stimulation, acutely inhibit adenylyl cyclase, so we hypothesized that the liberated $G_{\beta\gamma}$ subunits were responsible for adenylyl cyclase superactivation. To test this, we expressed the retinal $G_{\beta\gamma}$ scavenger, phosducin, along with the human δ-opioid receptor in CHO cells. Results from these experiments implicated a vital role for $G_{\beta\gamma}$, but to definitively confirm this, we created a stable cell line expressing both the human δ-opioid receptor and another $G_{\beta\gamma}$ scavenger, α-transducin. Results from these cells definitively verified our $G_{\beta\gamma}$ hypothesis.

Once this was established, we begin envisioning a signal transduction pathway, starting with δ-opioid receptor activation, propagated through $G_{\beta\gamma}$ subunits. We imagined that the result of this pathway might involve kinase activation and phosphorylation of adenylyl cyclase. Therefore, we next investigated the possibility that adenylyl cyclase VI could be phosphorylated by kinases activated in response to human δ-opioid receptor activation.

CHAPTER 5: THE ROLE OF KINASES IN ADENYLYL CYCLASE SUPERACTIVATION

INTRODUCTION

Once we established that G-protein βγ subunits were required for adenylyl cyclase superactivation, we began investigating possible downstream effectors that might lead to long-term compensatory changes in adenylyl cyclase activity. Physiological processes that lead to increases in adenylyl cyclase activity depend on the adenylyl cyclase isoform, but can include such factors as free intracellular calcium, activated $G_{\alpha s}$, and phosphorylation of certain adenylyl cyclases by particular kinases. Considering the pervasiveness of modulation through phosphorylation, we hypothesized the most probable modification was phosphorylation of adenylyl cyclase by a kinase downstream of human δ-opioid receptor stimulation. Because many $G_{\beta\gamma}$-mediated signaling pathways lead to protein kinase activation, the complexity inherent in this hypothesis has made determining the relevant kinase(s) difficult. Fortunately, we had some insight into the issue from reexamination of previous data and data obtained using another cell line.

When the human δ-opioid receptor was stably transfected into Chinese hamster ovary cells in 1994, creating hDOR/CHO cells, it was subsequently also stably transfected into mouse fibroblast B82 cells, creating the hDOR/B82 cell line. Before the human δ-opioid receptor was stably expressed in mouse fibroblast B82 cells, however, scientists in this laboratory noted the inability of B82 cells expressing the muscarinic M_2 acetylcholine receptor to exhibit carbachol-mediated adenylyl cyclase superactivation. As this receptor was a $G_{\alpha i/o}$-coupled receptor that had previously been shown to mediate a cAMP overshoot in other cells (much like the δ-opioid receptor), B82 cells were

transfected with the human δ-opioid receptor to determine if the problem lay with the receptor or with the cell line. At the time, these cells were characterized by [^{3}H]-NTI saturation binding and for their ability to inhibit forskolin-stimulated cAMP formation. We decided to further characterize these cells to determine if they might offer some insight into the mechanism of adenylyl cyclase superactivation. We began by measuring the SNC80-mediated inhibition of forskolin-stimulated adenylyl cyclase. Next, we investigated the ability of SNC80 to stimulate PLCβ and of these cells to exhibit adenylyl cyclase superactivation. Finally, we determined which adenylyl cyclase isoforms were expressed in B82 cells to ascertain a correlation between adenylyl cyclase isoform specificity, activation of PLCβ, and adenylyl cyclase superactivation.

To investigate a relationship between superactivation and phosphorylation of adenylyl cyclase we first needed to demonstrate a change in the phosphorylation status of adenylyl cyclase in CHO cells expressing the human δ-opioid receptor. We attempted reveal an increase in adenylyl cyclase phosphorylation under the same agonist pretreatment conditions as those that cause superactivation. As in the characterizations outlined in previous chapters, we also determined the naltrindole sensitivity of this phosphorylation and a time-course to demonstrate phosphorylation in response to chronic, but not acute agonist pretreatment. Then we set out to inhibit this phosphorylation with various kinase inhibitors.

Once human δ-opioid receptor-mediated phosphorylation of adenylyl cyclase in CHO cells was established, we hypothesized a link between such [^{32}P] incorporation and superactivation. We decided to test this by determining which kinase(s) were responsible

for phosphorylation by using selective kinase inhibitors and demonstrating an inhibition of agonist-mediated [^{32}P] incorporation. We used inhibitors of the major second messenger-activated kinases, cAMP-dependent protein kinase (protein kinase A or PKA), protein kinase C (PKC), and calcium calmodulin-dependent protein kinase (CaMK). Within each of these classes, we determined adenylyl cyclase phosphorylation using two structurally different inhibitors to verify inhibition of the kinases of interest.

Next, we examined several of these inhibitors in the adenylyl cyclase superactivation assay. The goal here was, of course, to correlate the inhibitors that resulted in loss of phosphorylation with those that inhibited adenylyl cyclase superactivation. Upon investigation into the literature, we discovered a reference to the serine/threonine kinase typically understood to function in the mitogen-activated protein kinase (MAPK) pathway, p74, cRaf, or Raf-1 (Tan, et al., 2001). It was shown that this kinase could phosphorylate a certain adenylyl cyclase isoform and increase the activity of the adenylyl cyclase. An appealing aspect of this interaction was the array of physiological processes capable of activating Raf-1. As will be outlined below, we have amassed data that were initially difficult to interpret, but that could be easily explained by the involvement of Raf-1. Due to its relatively poorly characterized function in G-protein-coupled receptor signal transduction, we did not initially investigate a role for Raf-1 in either adenylyl cyclase phosphorylation or superactivation. However, we have begun to establish what appears to be an important, potentially vital, function for Raf-1 in these processes. This is the topic of our current work, which will likely bear some long-sought breakthroughs concerning the molecular mechanism of adenylyl cyclase

superactivation in CHO cells expressing the human δ-opioid receptor, and hopefully in other systems as well.

Due to these findings, we currently hypothesize that adenylyl cyclase phosphorylation by Raf-1 kinase mediates adenylyl cyclase superactivation. We believe that the routes to activation of Raf-1 kinase begin with chronic stimulation of the human δ-opioid receptor, but diverge into multiple parallel pathways, each capable of Raf-1 activation and subsequent adenylyl cyclase superactivation. Furthermore, we believe that inhibition of any one of these pathways results in shunting the signal through a parallel pathway, thereby making complete inhibition of adenylyl cyclase superactivation possible only by inhibiting more than one pathway.

METHODS

[^{32}P] Incorporation into ACVI

hDOR/CHO cells were plated on 10 cm plates and grown to 90% confluency. They were subsequently incubated in phosphate free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% dialyzed fetal calf serum for 45 minutes and labeled with [^{32}P]-orthophosphate (NEN, 3000 Ci/mmol, 200 μCi/ml) for 1 hour. After the metabolic labeling, the incubation continued in the presence of [^{32}P]-containing medium alone (control), 1 μM SNC80, or 1 μM SNC80 supplemented with 1 μM naltrindole. For the kinase inhibitor experiments, the compounds were added along with the 1 μM SNC80. After a 24 hour incubation, the cells were washed twice with phosphate buffered

saline and scraped into ice cold homogenization buffer (50 mM Tris, 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaF and 10 mM Na-pyrophosphate supplemented with 10 µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), and 100 nM Na-orthovanadate (and 10 nM okadaic acid immediately before use). The cells were centrifuged at 25,000 rpm for 30 min and the pellet resuspended in 1 ml radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Igepal CA-630, 0.5% Triton X-100, 0.2% digitonin, 5 mM EDTA, 10 mM NaF, 10 mM beta-glycerol-phosphate with 10 µl/ml protease inhibitor cocktail, with 10 nM okadaic acid and 100 nM Na-orthovanadate added immediately before use. The solution was incubated on ice for 3 hours then centrifuged at 40,000 rpm for 30 min. The lysate was pre-cleared by incubation in the presence of 1 µg preimmune rabbit immunoglobulin G and 10 µl protein A-agarose for 1 hour, then centrifuged at 3000 rpm. The pre-cleared lysate was incubated overnight with adenylyl cyclase V/VI specific antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For the lysate in a third plate (peptide antagonism), the antibody was pre-incubated with 50 µl blocking peptide (Santa Cruz Biotechnologies) for 4 hours. 10 µl protein A-agarose was added and the mixture incubated on ice with gentle rocking for 3 hours, centrifuged at 3000 rpm for 5 minutes, and washed three times with 10 minute incubations in RIPA wash buffer (RIPA buffer with detergent concentrations reduced to 0.075% Triton X-100, 0.05% Igepal CA-630 and 0.1% digitonin) in the presence of protease and phosphatase inhibitors as outlined above. The immunoprecipitate was eluted from the final pellet by incubating with 10 µl glycine buffer, pH=2.3. The mixture was neutralized with 5 µl neutralization buffer and boiled

with 15 µl of 2X Laemmli sample buffer for 5 min. The immunoprecipitate was resolved on a 7.5% denaturing polyacrylamide gel. The gel was stained with Coomassie blue, destained, dried, and subjected to autoradiography. Both protein staining and autoradiography obtained two protein bands of approximately 130 and 200 kDa. The 200-kDa band was presumably the glycosylated form of the adenylyl cyclase VI protein (deduced MW=130 kDa), because only the 130 kDa band was apparent after *N*-aminoglycosidase-F treatment of the immunoprecipitate (data not shown). The protein content of the Coomassie stained 200 kDa band and the [^{32}P] incorporation (autography film) were quantified using the Arcus II scanning densitometer with the Documax OneDScan software for the IBM-compatible PC (Scanalytics, Billerica, MA).

Adenylyl cyclase superactivation after pretreatment with kinase inhibitors

hDOR/CHO cells were seeded at 65,000 cells per well in 24-well plates and grown until 80-90% confluent in a humidified incubator (5% CO_2, 95% air), about 48 hours. The growth medium was aspirated and replaced with serum-free IMDM. Either the kinase inhibitor (0.5 µM Calmidazolium, 5 µM KN-93, 0.1 µM Calphostin C, 1 µM U0126, or 10 µM GW5074) or equivalent solvent in IMDM was added to the wells 30 minutes before the addition of 100 nM SNC80. Kinase inhibitors or equivalent solvent was also added to wells for counts treated in parallel. Cells were then subjected to the adenylyl cyclase superactivation and cAMP assay as described in the GENERAL METHODS.

RESULTS

Mouse fibroblast B82 cells stably transfected with the human δ-opioid receptor (hDOR/B82) were previously partially characterized. The expression of human δ-opioid receptors in these cells (B_{max}) was determined to be 234 ± 15 fmol per million cells in the [^{3}H]-naltrindole saturation assay. Since then, we have investigated the ability of δ-opioid receptor agonists in these cells to activate PLCβ and to inhibit adenylyl cyclase. **Figure 29** shows the inhibition of forskolin-stimulated adenylyl cyclase activity by SNC80 in these cells. The data are displayed as pmol cAMP per millions cells. Of interest was the significantly lower maximal cAMP formation in these cells as compared with the Chinese hamster ovary cells. Whereas hDOR/B82 have an E_{max} value of 21 ± 0.6 pmol cAMP per million cells (n=2), hDOR/CHO cells exhibit 238 ± 62 pmol per million cells (n=8, data not shown). In spite of their limited ability to produce cAMP, hDOR/B82 cells still exhibited robust SNC80-mediated inhibition of cAMP

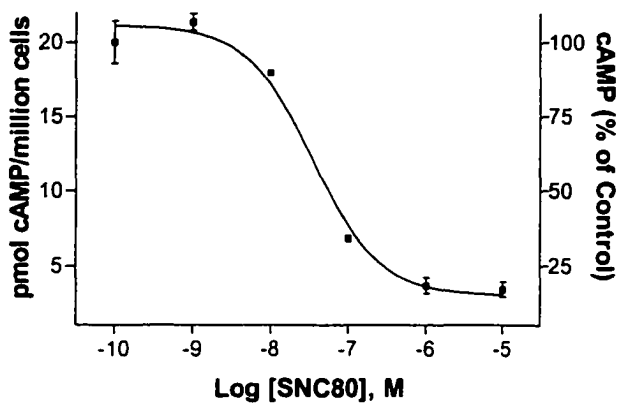

Figure 29: SNC80-inhibition of adenylyl cyclase in hDOR/B82 cells

SNC80 inhibited 100 μM forskolin-stimulated cAMP formation in hDOR/B82 cells to 21 ± 0.6 pmol per million cells with an EC_{50} of 34.9 ± 17 nM. When converted to a percent of control, this E_{max} was 15 ± 3% (n = 2).

formation, lowering forskolin-stimulated cAMP to 15 ± 3% of control. The EC_{50} for SNC80 in this assay was 34.9 ± 17 nM, which was about 25-fold less potent than SNC80 in hDOR/CHO cells under equivalent conditions.

We also examined SNC80-mediated IP_1 formation in these cells (**Figure 30**). In spite of our earlier belief that SNC80-mediated IP_1 formation was not possible in hDOR/B82 cells, it appeared that when the experiments were combined, there was a significant PLCβ stimulation. The intrinsic activity of SNC80 (E_{max}) was 123 ± 3% of control. While there was some scatter in the data, the 95% confidence interval of the regression E_{max} value (117% to 129%) does not contain the baseline control value of 100%, indicating statistically significant stimulation ($p<0.05$, $n=2$). The EC_{50} for SNC80 in this assay was 44.7 ± 130 nM, or about equipotent relative to SNC80 in the equivalent assay in hDOR/CHO cells. The basal unstimulated activity of these cells was 627 ± 31 CPM, not statistically different from the basal activity in hDOR/CHO cells (589 ± 900 CPM) (data not shown).

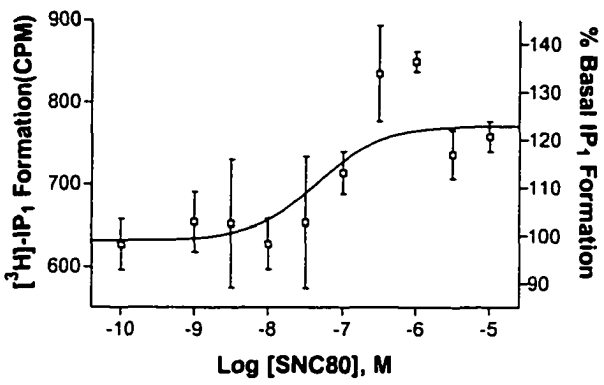

Figure 30: SNC80-mediated PLCβ stimulation in hDOR/B82 cells

SNC80 stimulated IP_1 formation in hDOR/B82 cells from 632 ± 27 to 771 ± 20 CPM with an EC_{50} of 44.7 ± 130 nM. When converted to a percent of control, this E_{max} was 123 ± 3% ($p<0.01$, $n = 2$).

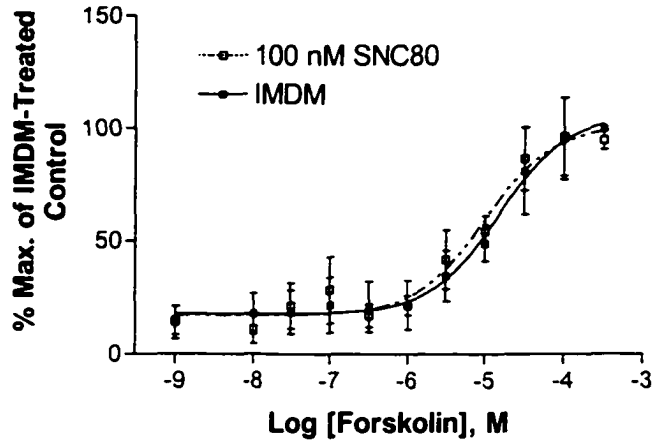

Figure 31: Absence of cAMP overshoot in hDOR/B82 cells

hDOR/B82 cells treated for 24 hours with 100 nM SNC80, then washed failed to exhibit adenylyl cyclase superactivation. There was no significant difference between any of the regression parameter in the two analyses ($p>0.50$, $n = 4$).

Figure 31 shows a forskolin concentration-response curve in hDOR/B82 cells under the same conditions that result in adenylyl cyclase superactivation in hDOR/CHO cells. In this assay, forskolin stimulated adenylyl cyclase with an EC_{50} of 15.1 ± 15 μM for IMDM-treated cells and 9.86 ± 9.0 μM for SNC80-treated cells. These figures were not significantly different from those observed in hDOR/CHO cells. The difference between these cell lines, however, was in the inability of the hDOR/B82 cells to exhibit adenylyl cyclase superactivation. When treated for 24-hours with 100 nM SNC80, then washed three times, hDOR/B82 cells do not exhibit increased (or decreased) forskolin-stimulated cAMP formation ($p>0.50$, $n=4$).

Due to the understood contribution of adenylyl cyclase isoform specificity in superactivation, we opted to determine the adenylyl cyclase isoforms expressed in B82 cells (Varga, et al., 1998). Using RT-PCR and transformation methodology (see Chapter 3 for methods), clones containing cDNA encoding adenylyl cyclase VII and IX were isolated (**Table 2**). The only forms of adenylyl cyclase shown to superactivate are I, V,

VI, and VIII. We concluded that adenylyl cyclase superactivation was not possible in hDOR/B82 cells because none of these adenylyl cyclase isoforms existed in B82 cells. As mentioned in Chapter 3, we determined that CHO cells express ACVI, a superactivatable adenylyl cyclase, and decided to further investigate the potential phosphorylation of this enzyme.

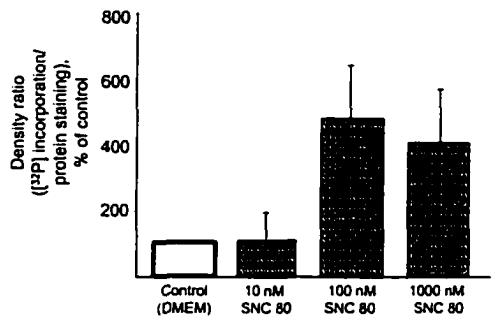

Figure 32: SNC80-mediated phosphorylation of ACVI
Whereas 10 nM SNC80 could not induce phosphorylation of ACVI over basal, 100 nM and 1 μM SNC80 significantly increased the [^{32}P] incorporation ($p<0.05$, $n = 2$).

We examined the phosphorylation of adenylyl cyclase VI in human δ-opioid receptor-expressing CHO cells after 4-hour treatment with different concentrations of SNC80 (0, 10, 100, and 1000 nM) (**Figure 32**). It can be seen that 10 nM SNC80 has no effect, whereas both 100 and 1000 nM SNC80 produce maximal phosphorylation of adenylyl cyclase VI. Next, we inhibited SNC80's ability to stimulate phosphorylation of adenylyl cyclase VI by including 1 μM naltrindole with the

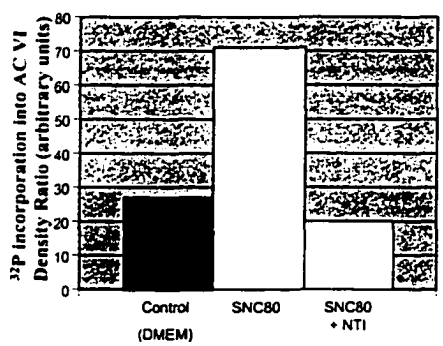

Figure 33: Naltrindole antagonism of SNC80-mediated ACVI phosphorylation
1 μM SNC80 significantly increased the relative [^{32}P] incorporation into ACVI, which was completely antagonized by co-incubation with 1 μM naltrindole (NTI).
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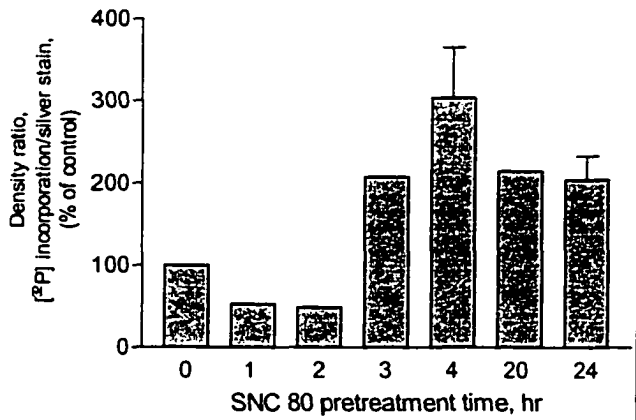

Figure 34: Time-course for SNC80-mediated ACVI phosphorylation

1 μM SNC80 significantly increased the relative [^{32}P] incorporation into ACVI over time. Maximal ACVI phosphorylation occurred by 4 hours (n = 7) and persisted for 24 hours (n = 3).

SNC80 for 4 hours (**Figure 33**). Additionally, we carried out a time-course for 1 μM SNC80-mediated phosphorylation of adenylyl cyclase VI. Maximal [^{32}P] incorporation was achieved by 4 hours (**Figure 34**).

At this point, we began to investigate the abilities of various selective kinase inhibitors to prevent adenylyl cyclase VI phosphorylation in hDOR/CHO cells. The kinases we were interested in were PKA, PKC, CaMK, and MAPK, as these were considered fairly ubiquitous and well-characterized signal transduction modulators. For PKA inhibition, we used 1 μM KT5720 or 2 μM H-89 (N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl). For PKC inhibition, we used 5 μM chelerythrine or 2 μM Gö6850 (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide). For calcium calmodulin-dependent protein kinase, we used 5 μM KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) or 0.5 μM calmidazolium. **Figure 35** displays the abilities of these kinase inhibitors to prevent SNC80-mediated adenylyl cyclase VI phosphorylation. The most evident conclusion was that both CaMK inhibitors, KN-93 and calmidazolium, completely blocked the increased

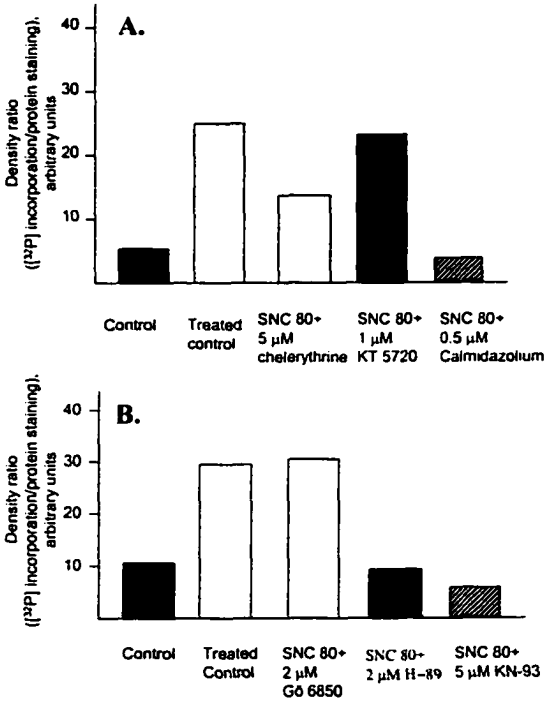

Figure 35: SNC80-mediated ACVI phosphorylation with kinase inhibitors

A. 1 μM SNC80 stimulated ACVI [^{32}P]-incorporation in 4 hours. This increase was modestly inhibited by co-pretreatment with chelerythrine (PKC inhibitor), but not affected by KT 5720 (PKA inhibitor). Phosphorylation was completely blocked by calmidazolium (calmodulin inhibitor).

B. Gö6850 (PKC inhibitor) had no effect, but both H-89 (PKA inhibitor) and KN-93 (calmodulin inhibitor) inhibited ACVI phosphorylation.

[^{32}P] incorporation. As for the other kinase inhibitors, the results were a bit equivocal. However, only one inhibitor from each group (H-89 or chelerythrine) appeared to modestly block phosphorylation. The other member of each group (KT5720 or Gö6850) had no effect.

We then examined the abilities of these and other similar kinase inhibitors to prevent adenylyl cyclase superactivation in hDOR/CHO cells. **Figure 36** demonstrates that PKC activation was not required for adenylyl cyclase superactivation. In this assay, the PKC activator, 100 nM phorbol-12-myristate-13-acetate (PMA), had no effect on forskolin-stimulated cAMP formation in hDOR/CHO cells. Additionally, in cells treated with the PKC inhibitor, calphostin C, SNC80 was still able to superactivate adenylyl cyclase (141% of maximally-stimulated IMDM control, preliminary results, n=1). Neither

calphostin C with PMA nor calphostin C alone had any effect on maximal cAMP formation relative to IMDM-treated cells.

We also examined the ability of the selective MAP kinase kinase (MEK) inhibitor, U0126, to affect SNC80-mediated adenylyl cyclase superactivation (**Figure 37**). 1 μM U0126 was included with 100 nM SNC80 for the 4-hour pretreatment. In this figure, untreated cells exhibited a significant cAMP overshoot of 360 ± 52% of IMDM-treated control (100 μM forskolin, $p<0.01$, n=2). Cells treated with U0126 also showed a significant cAMP overshoot of 311 ± 25% of IMDM-

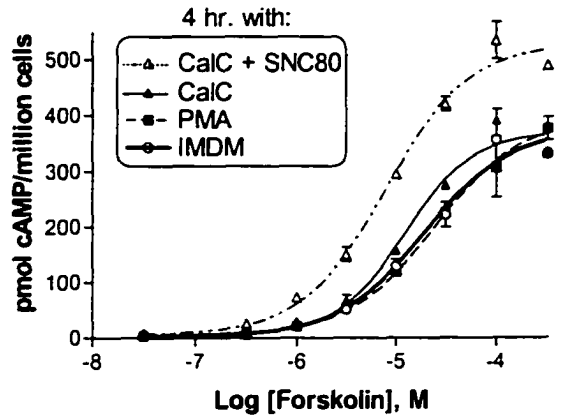

Figure 36: Adenylyl cyclase superactivation with calphostin C
hDOR/CHO cells demonstrated a 100 nM SNC80-mediated cAMP overshoot of 156 ± 38 pmol per million cells in the presence of the PKC inhibitor, 100 nM calphostin C (where calphostin C alone had no effect). Additionally, the PKC activator, 100 nM PMA, did not affect the maximal forskolin stimulation (n = 1).

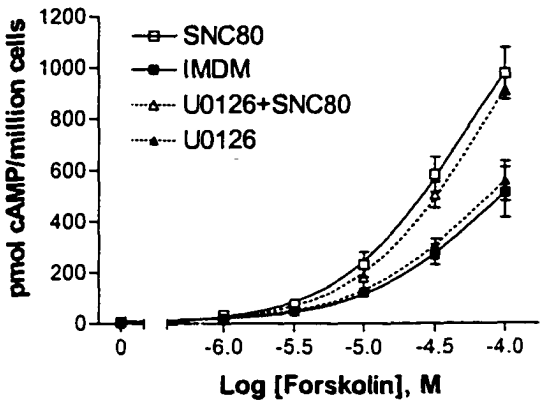

Figure 37: Adenylyl cyclase superactivation with U0126
hDOR/CHO cells demonstrated a 100 nM SNC80 cAMP overshoot (regression E_{max}) of 598 ± 390 pmol per million cells. In the presence of the MEK inhibitor, 1 μM U0126, there was no difference in the cAMP overshoot (603 ± 286). 1 μM U0126 alone had no effect (n = 2).

treated control (100 μM forskolin, $p<0.01$, n=2). 1 μM U0126 alone had no effect on forskolin-stimulated cAMP formation.

Next, we examined the CaMK inhibitor, calmidazolium, in the adenylyl cyclase superactivation paradigm. In **Figure 38A**, we noted that hDOR/CHO cells treated concomitantly with 0.5 μM calmidazolium exhibited a significantly increased maximal raw forskolin-stimulated cAMP formation (in the absence of SNC80) (669 ± 140 and

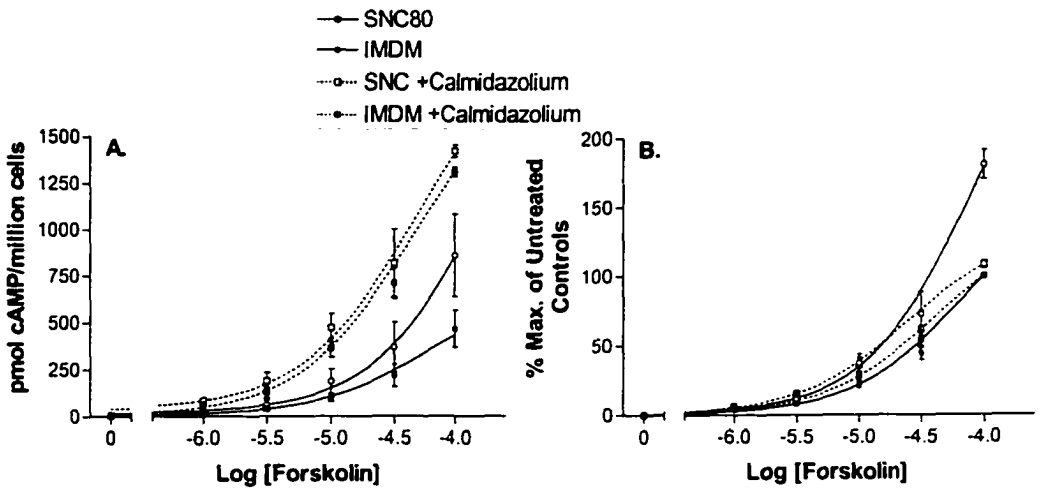

Figure 38: Adenylyl cyclase superactivation with calmidazolium

A. hDOR/CHO cells demonstrated a 100 nM SNC80 cAMP overshoot of 1353 ± 1600 pmol per million cells. In the presence of the calmodulin inhibitor, 0.5 μM calmidazolium, there was no cAMP overshoot (-152 ± 370 pmol per million cells), however, the cells treated with calmidazolium alone had a significantly increased E_{max} (1821 ± 109 pmol per million cells versus 669 ± 140 pmol per million cells for IMDM-treated cells) (n = 3). **B.** The same data converted to a percent of maximally stimulated control indicated that the cAMP overshoot was completely abolished by pretreatment with calmidazolium. Untreated cells exhibited an overshoot of 335 ± 43%, whereas calmidazolium-treated cells demonstrated only 135 ± 5.5% ($p>0.10$, n = 3).

1821 ± 109 pmol per million cells for IMDM- and calmidazolium-treated cells, respectively, $p<0.001$, n=3). Therefore, the data were normalized to each condition's (with and without calmidazolium) IMDM-treated control (**Figure 38B**). When plotted this way, 0.5 μM calmidazolium appears to completely inhibit adenylyl cyclase superactivation. While untreated cells exhibit a significant cAMP overshoot of 335 ± 43% of control ($p<0.05$), calmidazolium-treated cells exhibit a statistically insignificant overshoot of 135 ± 5.5% of control ($p>0.1$).

Finally, we recently began looking into the involvement of the MAP kinase kinase kinase, Raf-1, in adenylyl cyclase superactivation. We have examined its role using the selective Raf-1 kinase inhibitor, GW5074. Using this inhibitor, we have been able to demonstrate a significant inhibition of deltorphin-mediated adenylyl cyclase

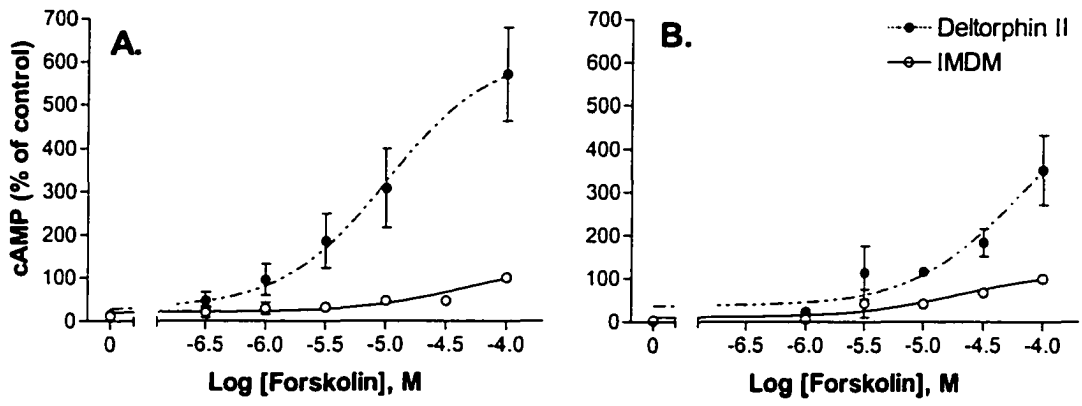

Figure 39: Adenylyl cyclase superactivation with GW5074

A. 4 hour 1 μM deltorphin treatment augments 100 μM forskolin stimulated cAMP formation to 570 ± 286 % of the control in hDOR/CHO cells (n=7). **B.** 30 minute pretreatment of the cells with 10 μM GW5074 attenuates chronic deltorphin-mediated adenylyl cyclase superactivation by 40% (351±197% of control, $p<0.01$, n=6).

superactivation (**Figure 39**). In this figure, hDOR/CHO cells were pretreated with 10 μM GW5074 for 30 minutes before the addition of 1 μM deltorphin II for 4 hours. The cAMP overshoot mediated by deltorphin II amounted to 571 ± 108% of maximally-stimulated IMDM control. Pretreatment with GW5074 resulted in a significant decrease in this value to 351 ± 81% of control ($p<0.05$, $n=7$). None of the other regression parameters were statistically different between the treatment groups.

DISCUSSION

B82 cells stably transfected with the human δ-opioid receptor were shown to express 234 ± 15 fmol of receptor per million cells. This figure represented about a 7-fold lower expression than current human δ-opioid receptor expression in CHO cells. However, the original measurement of B_{max} in hDOR/CHO cells showed about 150 fmol of receptor per million cells, and these cells were capable of robust and consistent adenylyl cyclase superactivation. Such abundant expression by each of these cells likely resulted in a vast receptor reserve, thereby creating a saturated E_{max} in every situation. Evidence of this is the near complete SNC80-mediated inhibition of forskolin-stimulated cAMP formation and potent EC_{50} (about 35 nM) in hDOR/B82 cells (**Figure 29**).

As shown in **Figure 31**, B82 cells expressing the human δ-opioid receptor (hDOR/B82 cells) did not exhibit of adenylyl cyclase superactivation, exactly like their M_2-acetylcholine receptor-transfected counterparts. We had earlier considered the possibility that adenylyl cyclase superactivation may have been mediated by protein kinase C, subsequent to PLCβ activation. Therefore, we examined the SNC80-mediated

IP_1 formation in hDOR/B82 cells (**Figure 30**). We were able to demonstrate a small, but significant ($p<0.05$) PLCβ activation. This finding gave us cause to believe that either the PLCβ response was not involved in adenylyl cyclase superactivation, or that additional cellular components were necessary and not expressed in B82 cells. To examine this question further, we determined the adenylyl cyclase isoforms expressed in B82 cells.

We identified adenylyl cyclase types VII and IX in B82 cells. This was significant because, of the nine isoforms examined, only I, V, VI, and VIII were shown to be capable of superactivation. Because neither adenylyl cyclase VII nor adenylyl cyclase IX were on this list, we suspected that hDOR/B82 cells could not exhibit a cAMP overshoot for lack of an appropriate adenylyl cyclase. However, preliminary results obtained by stably transfecting adenylyl cyclase VI into hDOR/B82 cells indicated otherwise (data not shown). We tentatively concluded that there was more molecular machinery missing in B82 cells than simply the correct adenylyl cyclase and proceeded to investigate the role that phosphorylation played in adenylyl cyclase superactivation in hDOR/CHO cells.

The simplest method to ascertain whether a kinase was required and, if so, which one was to employ selective kinase inhibitors for the well-characterized enzymes and then measure adenylyl cyclase VI phosphorylation and superactivation. We examined the possible, though unlikely, involvement of PKA. Activation of the human δ-opioid receptor resulted in inhibition of adenylyl cyclase. Even after chronic treatment, intracellular cAMP concentrations have been returned to just pretreatment levels, and no greater, as indicated in **Figure 2**. During the formation of adenylyl cyclase superactivation, the concentration of cAMP never increases; we found it improbable that

PKA activation was really a factor in the production of adenylyl cyclase superactivation. This hypothesis was confirmed in **Figure 35**. The highly selective PKA inhibitor, KT5720, did not affect ACVI phosphorylation, whereas the non-selective inhibitor, H-89, did show an inhibition. H-89 has been shown to inhibit, among other kinases, CaMK. This aspect of H-89's mechanism of action has become important for explaining the partial inhibition noted. Therefore, we do not believe that activation of PKA is necessary for adenylyl cyclase superactivation.

There were many accounts of δ-opioid-mediated PKC activation, although this pathway tends to be complicated by the various forms of PKC and their manners of regulation (see Chapter 1). Whereas chelerythrine has been shown to inhibit members of the atypical PKC family, Gö6850 has not. These factors were important, as chelerythrine inhibited SNC80-mediated phosphorylation of ACVI, but Gö6850 had no effect. Due to the differences in their selectivity, this finding indicated a putative involvement of the aPKCs (e.g. PKCζ) in ACVI phosphorylation, and possibly adenylyl cyclase superactivation.

Finally, the calcium calmodulin-dependent protein kinase inhibitors, calmidazolium and KN-93, were examined. As seen in **Figure 35**, both of these inhibitors blocked SNC80-mediated [^{32}P] incorporation into ACVI. These inhibitors act by antagonizing calcium calmodulin (CaM), not by simply inhibiting the CaM kinase. Therefore, the noted inhibition was evidence for a central role for calmodulin and its downstream effectors, including, but not limited to, CaMK. Furthermore, these results

indicated that the inhibition seen by H-89 could have been explained by its ability to inhibit CaMK activation.

Next, we examined kinase inhibition in the adenylyl cyclase superactivation assay. Because different selective inhibitors were used in the phosphorylation assay, the inhibition of adenylyl cyclase superactivation by various kinase inhibitors painted a slightly different picture, although a central role for calmodulin or CaMK appeared to be consistent.

To examine PKC involvement, we used the phorbol ester, phorbol-12-myristate-13-acetate (PMA), to selectively activate the PKC and calphostin C to inhibit it. It should be noted that PMA serves to mimic diacylglycerol and only stimulates PKC isoforms that are capable of DAG-mediated activation. Whereas the conventional and novel PKCs fall into this category, DAG does not activate the atypical PKCs (aPKC). Furthermore, calphostin C acts analogously by competing at the same DAG (or PMA) site. **Figure 36** showed that pretreatment with either calphostin C or PMA alone did not affect forskolin-stimulated cAMP formation (relative to IMDM pretreatment), and that co-pretreatment with SNC80 and calphostin C still resulted in a significant cAMP overshoot ($p<0.001$, $n=1$). Because these preliminary results were ultimately negative, we did not extensively characterize this putative interaction. However, a negative result in this assay simply excluded conventional PKC (cPKC) and novel PKC (nPKC), but for the reasons mentioned above, aPKC may still have been involved. In fact, the results from the ACVI phosphorylation assay using chelerythrine and Gö6850 indicated a putative role for the

aPKCs. As will be outlined, our overall hypothesis and future studies have incorporated these interpretations.

As outlined in the Chapter 1, activation of the mitogen activated protein (MAP) kinase pathway by the δ-opioid receptor has been demonstrated. To inhibit this pathway, we used U0126, which was a selective inhibitor of MEK (the kinase that phosphorylates and activates MAPK). Our results indicated that 1 μM U0126 did not affect adenylyl cyclase superactivation (**Figure 37**). Therefore, this inhibitor was not examined in the phosphorylation assay.

We investigated the role of calmodulin using 0.5 μM calmidazolium- a concentration that was shown to completely inhibit [^{32}P] incorporation into ACVI. hDOR/CHO cells exhibited an interesting phenomenon when pretreated with this inhibitor, either with or without 100 nM SNC80. **Figure 38A** shows the raw data in pmol cAMP per million cells. The cells exposed to calmidazolium alone made significantly more forskolin-stimulated cAMP than IMDM-treated cells, and cells pretreated with both calmidazolium and SNC80 made significantly more cAMP than both IMDM- and SNC80-pretreated cells ($p<0.001$, $n=3$). However, cells treated with both calmidazolium and SNC80 showed no significant increased in forskolin-stimulated cAMP formation over cells treated with calmidazolium alone. In other words, calmidazolium raised the baseline, but completely attenuated the cAMP overshoot. When the baselines were normalized, as shown in panel B, this conclusion becomes evident. Because the baseline was shifted so dramatically, we could not draw any definitive conclusions about the

involvement of calmodulin in adenylyl cyclase superactivation, however, these results do indicate that calmodulin may play an important role in the compensatory response.

An extensive literature search produced an account of ACVI phosphorylation and activation by Raf-1 kinase, a member of the MAP kinase cascade. This was intriguing, as opioid receptor-mediated Raf-1 activation (and subsequent MAP kinase activation) has been shown to occur through many diverse pathways, many of them potentially occurring in hDOR/CHO cells. We had considered the MAP kinase pathway, hypothesizing the potential involvement of the MAP kinase itself. As stated, the inhibitor we used to examine the involvement of MAP kinase, U0126, inhibited MEK, the kinase just upstream of MAPK. If Raf-1, the kinase just upstream of MEK, were important, U0126 would not have had any effect, just as we observed. Therefore, we looked into the ability of the selective Raf kinase inhibitor, GW5074, to affect adenylyl cyclase superactivation (**Figure 39**). Although this inhibitor did not shift the baseline like calmidazolium, the raw forskolin-stimulated cAMP that was formed from experiment to experiment was variable and, therefore, impossible to combine. The significant attenuation of adenylyl cyclase superactivation in the presence of GW5074 strongly implicated a central role for Raf-1 kinase. Our investigations into Raf-1 have been ongoing and current preliminary experiments continue to link Raf-1 with human δ-opioid receptor-mediated adenylyl cyclase superactivation in CHO cells.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

The mechanism of adenylyl cyclase superactivation has proven quite elusive, even though it has been consistently investigated since its discovery over twenty-five years ago. It appears to occur universally in tissues that endogenously express opioid (and presumably many other $G_{i/o}$-coupled) receptors. As a phenomenon, adenylyl cyclase superactivation has been linked to chronic opioid administration *in vivo* on many occasions. An important site of action of opioids in the brain is the locus coeruleus (Duman, et al., 1988; Nestler, et al., 1994). The neurons in this structure fire at a rate proportional to their intracellular cAMP concentrations (Alreja and Aghajanian, 1993; Nestler, et al., 1999). Stimulated opioid receptors in these cells activate G-protein-coupled inwardly rectifying potassium channels (GIRK), and the acute inhibition of adenylyl cyclase decreases the intracellular cAMP, thereby deactivating non-selective cation channels (Alreja and Aghajanian, 1994). Upon chronic treatment with opioids, the firing rate of the neurons in the locus coeruleus returns to pretreatment levels due to adenylyl cyclase superactivation and the normalization of cAMP concentrations. Then, during withdrawal, the resultant cAMP overshoot ensues and the firing rate of locus coeruleus neurons overshoots as well (Nestler, 1992). This increased firing rate has been shown to contribute to the physical symptoms of opioid withdrawal (Nestler, 1992). Similar responses have been demonstrated in other important brain regions as well. These include the nucleus accumbens, the ventral tegmental area, the dorsal raphe nucleus, and the periaquaductal grey matter (Widnell, et al., 1996; Bonci and Williams, 1997; Punch, et al., 1997; Jolas, et al., 2000). These structures are important in mediating opioid

addiction and withdrawal, and have been shown to be significant for the effects of many drugs of abuse (Nestler and Aghajanian, 1997).

We set out to characterize the mechanism of δ-opioid receptor-mediated adenylyl cyclase superactivation in Chinese hamster ovary cells, however, this phenomenon is by no means limited to this cell line. Adenylyl cyclase superactivation and the resultant cAMP overshoot typically occur in tissues chronically exposed to agonists acting at a variety of $G_{\alpha i/o}$-coupled receptors. However, we are studying adenylyl cyclase superactivation mediated by the human δ-opioid receptor, as it seems likely that this receptor may become a mainstay of analgesic therapy in the future. Adenylyl cyclase superactivation, originally demonstrated more than twenty-five years ago (Sharma, et al., 1975a), has been implicated in the physiological processes necessary for the development of tolerance to drugs and the physical withdrawal syndrome that often follows. We have chosen to investigate the elusive molecular mechanism of adenylyl cyclase superactivation by stably expressing the human δ-opioid receptor in Chinese hamster ovary cells (hDOR/CHO cells). As opposed to studying the development of tolerance and withdrawal in the whole animal or even isolated brain tissue, hDOR/CHO cells allow much more control over the environment in which this phenomenon occurs. This important property has allowed us to definitively determine some key members of the signal transduction cascade that results in adenylyl cyclase superactivation.

In spite of the twenty-five years that have elapsed since its discovery, the precise mechanism of adenylyl cyclase superactivation in any one tissue has never been fully described. However, much progress has been made using transfected cell lines. We

believe that hDOR/CHO cells constitute a realistic model representative of naturally occurring tissues that innately exhibit this compensatory response. Evidence for this lies in some of the characteristics observed in these cells, as outlined earlier in Chapter 3. Many $G_{\alpha i/o}$-coupled receptors, including the human δ-opioid receptor, activate phospholipase Cβ through liberated $G_{\beta\gamma}$ subunits in intact, untransfected systems (Murthy and Makhlouf, 1996). The adenylyl cyclase superactivation in hDOR/CHO cells was pertussis toxin sensitive, as all superactivatable systems that have been investigated have been shown to be (Thomas and Hoffman, 1992; Avidor-Reiss, et al., 1995; Avidor-Reiss, et al., 1996; Ammer and Schulz, 1997; Rhee, et al., 2000; Rubenzik, et al., 2001). hDOR/CHO cells require five hours to reach maximal adenylyl cyclase superactivation, which is considered a relatively long time-course for a cellular response that does not involve novel protein synthesis. Many compensatory responses, such as agonist-mediated homologous receptor desensitization and internalization, typically reach equilibrium in a matter of minutes (Trapaidze, et al., 1996; Gaudriault, et al., 1997). As a result, it has been quite difficult to link these fast-acting processes with physiological responses to the same chronic agonists *in vivo*. Further evidence that these processes are unrelated lies in the observation that morphine, which causes adenylyl cyclase superactivation in every μ- or δ-opioid model examined, often fails to internalize or desensitize the receptor (Sim, et al., 1996; Selley, et al., 1997). Any model of tolerance and withdrawal should at least approach the time scale over which these processes occur in an animal. Additionally, novel protein synthesis is not required for adenylyl cyclase superactivation in hDOR/CHO cells. People have investigated adenylyl cyclase superactivation in whole

brain, isolated brain regions, bowel, primary cell cultures, endogenous $G_{\alpha i/o}$-coupled receptor-expressing cell lines, and a variety of receptor- transfected cell lines. Of these diverse systems, only a handful exhibited a modest requirement for de novo protein synthesis, whereas more typically, the cellular machinery for mediating adenylyl cyclase superactivation seemed to be previously expressed (Mukherjee, et al., 1976; Ammer and Schulz, 1993; Avidor-Reiss, et al., 1995; Shimizu, et al., 1996; Palmer, et al., 1997; Nakagawa, et al., 1998). It appears likely that whatever these cellular constituents are, they play important roles in the maintenance of normal cellular physiology and can be recruited as required for responses such as adenylyl cyclase superactivation. Despite the shortcoming that Chinese hamster ovary cells are not of human origin or an excitable tissue, measurable human δ-opioid receptor-mediated responses in these cells seem to mimic much of what others have observed in many endogenously opioid receptor-expressing systems.

We demonstrated a requirement for G-protein βγ subunits in the formation of adenylyl cyclase superactivation. Other groups investigating adenylyl cyclase superactivation in diverse tissues made this observation more significant by reaching the same conclusion. It seems likely, however, that $G_{\beta\gamma}$ subunits play different roles depending on the tissue. The expression of completely different profiles of enzymes and proteins in cells of assorted origins makes this assertion inevitable. For example, we know that different adenylyl cyclase isoforms respond differently to the same mediators. Whereas some $G_{\beta\gamma}$ subunits directly interact with ACV and ACVI to inhibit their activity, $G_{\beta\gamma}$ subunits have been shown to conditionally stimulate ACII, ACIV, and ACVII. This

helps explain why systems that express combinations of ACII, ACIV, or ACVII superactivate differently from those expressing ACV or ACVI. The necessity for $G_{\beta\gamma}$ subunits, however, appears to be consistent.

The other factor that appears to be consistent in adenylyl cyclase superactivation is the involvement of protein kinases and phosphorylation. Again, exactly which kinases and their roles depend on the system, but the involvement of PKC has been documented in a number of models. In hDOR/CHO cells, opioid-mediated activation of phospholipase Cβ and the subsequent production of diacylglycerol lead to protein kinase C activation. Indeed, PKC has been shown to be important for opioid receptor desensitization, internalization, and down-regulation (Pak, et al., 1997; Xiang, et al., 2001). Additionally, PKC has been shown to be important for superactivation in some systems by using chelerythrine (Wang, et al., 1996; Aley and Levine, 1997; Chakrabarti, et al., 1998b; Kramer and Simon, 1999). The activation of PKC in these systems has a similar time-course of activation as the development of *in vivo* opioid tolerance and dependence (Fundytus and Coderre, 1996). We have likewise indicated an important function for PKC in ACVI phosphorylation using chelerythrine. Due to the differential results obtained using chelerythrine and Gö6850, we believe that it is the atypical, diacylglycerol- and calcium-insensitive PKC isoforms (i.e. PKCξ). This hypothesis has implications for how the human δ-opioid receptor might mediate adenylyl cyclase superactivation, as outlined below.

The activation of PKA is sometimes vital for the formation and maintenance of opioid tolerance and dependence *in vivo*, however, its role in adenylyl cyclase

superactivation is unclear, as intracellular cAMP never rise above normal physiological concentrations during chronic opioid exposure. Rather, adenylyl cyclase is acutely inhibited and eventually recovers to perfectly compensate for the inhibition. Intracellular cAMP concentrations are only accentuated during withdrawal and the cAMP overshoot. It is probably during this phase that PKA becomes activated and mediates its effects on neuronal plasticity and the formation of opioid dependence. We were unable to demonstrate a clear function for PKA in either ACVI phosphorylation or superactivation.

We did, however, reveal a central role for either calcium calmodulin or CaMK using two different inhibitors. Others have likewise demonstrated the importance of calmodulin or CaMK in opioid-mediated *in vivo* tolerance (Fan, et al., 1999). Calmodulin activation might occur through PLCβ activation and the subsequent increases in inositol-1,4,5-trisphosphate and calcium. It is possible that activated calmodulin associates with and activates CaMK. There are additional routes of $G_{i/o}$-coupled receptor-mediated activation of CaMK as well, such as CaMKK- and PKA-mediated phosphorylation and calcium-independent autophosphorylation (Colbran, 1992; Soderling, 1999). Because the inhibitor that we used (calmidazolium) was merely a calmodulin antagonist and not a selective CaMK inhibitor, it likely prevented all calmodulin-dependent downstream effects, including the transactivation of a tyrosine kinase receptor as outlined in Chapter 1.

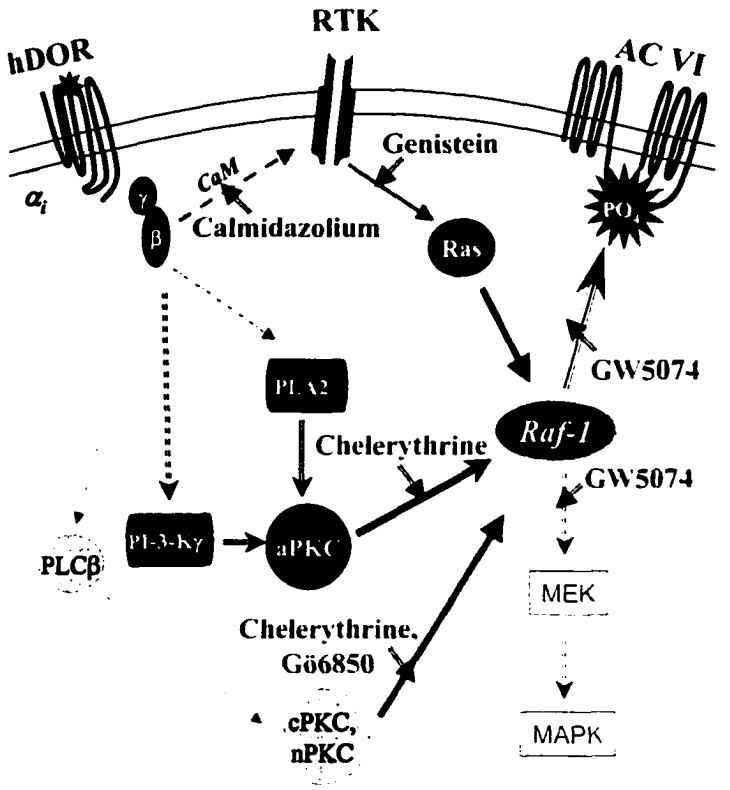

Figure 40: Hypothesis for adenylyl cyclase superactivation in hDOR/CHO cells
Stimulation of the human δ-opioid receptor in CHO cells liberates $G_{\beta\gamma}$ subunits, which can interact with many effectors. These include PLCβ, PI-3K, PLA_2, and, in conjunction with calmodulin, receptor tyrosine kinases. Whereas PLCβ activation results in the stimulation of the cPKCs and nPKCs, PI-3-Kγ and PLA_2 activation can stimulate the aPKCs. All of these PKC types can phosphorylate and activate Raf-1. Additionally, transactivation of the platelet-derived growth factor receptor (RTK) sends it signal through Raf-1 on its way to MAP kinase. Some inhibitors of these pathways are indicated.

In synthesizing all of these ideas, we have devised a hypothesis that incorporates a putative molecular mechanism for adenylyl cyclase superactivation in CHO cells (**Figure 40**). Based on our and other groups' observations, the most inclusive theory for the

mechanism implicates the newly characterized $G_{i/o}$-mediated MAP kinase cascade. Because MEK (a MAPKK) inhibition using U0126 had no effect on adenylyl cyclase superactivation, and likewise has not affected other superactivatable systems (Tso and Wong, 2001), a member of the MAPK-cascade that might be involved would have to be upstream of MEK (MAPKK). Raf-1 kinase, a MAPKKK, fits this profile. Additionally, Raf-1 has been shown to phosphorylate and activate ACVI (Tan, et al., 2001). We therefore hypothesize that Raf-1 is integrally involved in the formation of superactivated adenylyl cyclase in CHO cells. To this end, we have demonstrated that selective inhibition of this kinase by GW5074 attenuates adenylyl cyclase superactivation

Contrary to what was once the understood role for a MAPKKK, recent evidence suggests that Raf-1 activation does not always lead to ERK (a MAPK) activation (Osada, et al., 1999; Self, et al., 2001). Whereas another member of the Raf family, B-Raf, strongly interacts with and activates MEK, Raf-1 only weakly stimulates MEK and, therefore, MAPK. It is thought that the role of Raf-1 may be to transduce signals originating from tyrosine kinases to effectors outside the nucleus. Raf-1 can be activated by various isoforms of the small G-protein called Ras, members of which have been shown to colocalize with Raf-1 in cholesterol-rich lipid rafts or caveolae (Reuther and Der, 2000; Prior and Hancock, 2001). Further support for our hypothesis that Raf-1 activation causes adenylyl cyclase superactivation lies in the observation that under chronic agonist treatment conditions, $G_{i/o}$-protein-coupled receptors, their cognate G-proteins, and adenylyl cyclase all can be found colocalized in detergent-insoluble caveolae (Schwencke, et al., 1999; Bayewitch, et al., 2000; Ostrom, et al., 2001). It is

believed that caveolin, the major constituent of caveolae acts as a scaffold upon which signal transduction cascades are built. If adenylyl cyclase superactivation occurs through Raf-1 activation, it is no surprise that all of these components end up in caveolae after chronic receptor activation.

$G_{\alpha i/o}$-protein-coupled receptor-mediated activation of Raf-1 can occur by many routes. The most understood is through a process of receptor transactivation. Activated G-protein-coupled receptors have been shown to induce ligand-independent tyrosine phosphorylation of growth factor receptors and subsequent Ras-dependent activation of Raf-1 (Daub, et al., 1996; Daub, et al., 1997; Herrlich, et al., 1998). Such transactivation has been shown to be mediated by $G_{\beta\gamma}$ subunits liberated from either $G_{\alpha i/o}$ or $G_{\alpha q}$ subunits (Crespo, et al., 1994; Lopez-Ilasaca, et al., 1997; Gutkind, 1998; Luttrell, et al., 1999; Montaner, et al., 2001). Another method of activation involves $G_{\beta\gamma}$-mediated activation of phosphatidylinositol-3-kinase γ (PI-3Kγ) (Leopoldt, et al., 1998). Activated PI-3Kγ can phosphorylate and activate the non-receptor tyrosine kinase, Src, which eventually leads to Raf-1 activation. There has even been a report of Src activation by a calcium-dependent non-receptor tyrosine kinase, Pyk2 (Dikic, et al., 1996). In this case, the calcium in hDOR/CHO cells could be supplied by IP_3-mediated calcium release from intracellular stores. Finally, a rather uncharacterized novel mechanism of receptor tyrosine kinase transactivation has been shown to occur through G-protein-coupled receptor-mediated activation of membrane metalloproteases. These enzymes can be transactivated in a calcium calmodulin-dependent manner. Once activated, they cleave growth factor precursors in the membrane, thereby releasing ligands for receptor tyrosine

kinases such as the epidermal and platelet-derived growth factor receptors, where the latter are expressed in CHO cells (Oak, et al., 2001). Transactivation of these receptors results in the well-described ERK1/2 (MAPK) cascade that includes the activation of Raf-1. These pathways are summarized in **Figure 40**.

In this figure, we have outlined the pathways and some selective inhibitors of these pathways. These inhibitors can be used to investigate the contribution of each putative route of Raf-1 activation.

We have preliminary results demonstrating that GW5074 not only attenuates adenylyl cyclase superactivation, but also definitively inhibits Raf-1, as indicated by total MAPK and deltorphin-mediated phosphorylated MAPK in hDOR/CHO cells (**Figure 41**). In this immunoblot, 10 μM GW5074 had no effect on total MAPK protein, but noticeably inhibited MAPK phosphorylation (by MEK). The two bands represent ERK1 and ERK2 (p44

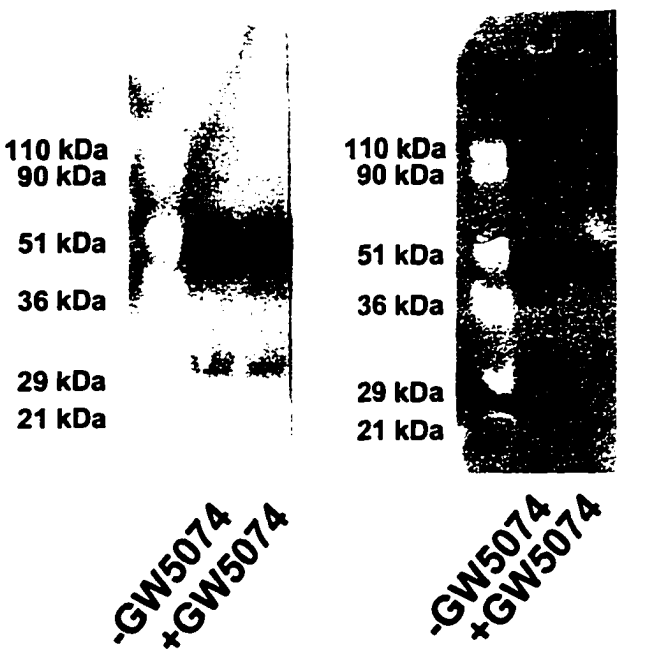

Figure 41: MAPK and phosphoMAPK immunoblot with GW5074

30 minute preincubation of hDOR/CHO cells with 10 μM GW5074 attenuates 1 μM deltorphin-mediated MAPK phosphorylation.

and p42). The assumption was that decreased MEK phosphorylation was a result of decreased Raf-1 activity.

Using some of the inhibitors indicated in **Figure 40**, we have preliminary evidence for multiple parallel pathways for Raf-1 activation. **Figure 42** shows adenylyl cyclase superactivation in the presence of the non-receptor tyrosine kinase inhibitor, genistein, or the pan-PKC inhibitor, chelerythrine, or both inhibitors. Whereas chelerythrine was able to partially inhibit SNC80-mediated phosphorylation of ACVI, it was not capable of inhibiting adenylyl cyclase superactivation in this representative

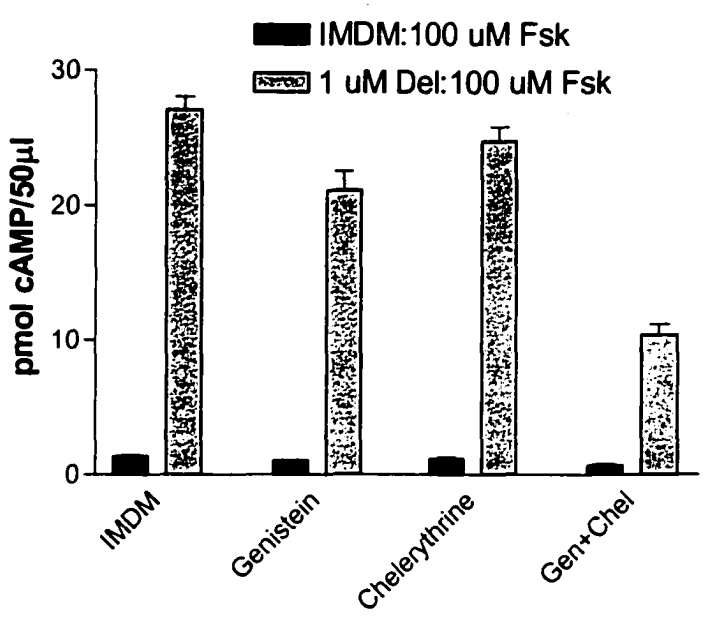

Figure 42: Adenylyl cyclase superactivation with TK and PKC inhibitors
30-minute pretreatment of hDOR/CHO cells with 100 µM genistein (non-receptor tyrosine kinase inhibitor) modestly attenuated the 1 µM deltorphin-mediated cAMP overshoot by 22%, whereas 5 µM chelerythrine (pan-PKC inhibitor) inhibited 9%. Pretreatment with both inhibitors decreased the cAMP overshoot by 62% (representative example of multiple experiments).

experiment. Genistein alone did cause a slight attenuation of the cAMP overshoot, but the combination of genistein and chelerythrine was able to limit the cAMP overshoot by about 50 percent. These results indicate that blocking one pathway results in the shunting of the signal through alternative parallel pathways and that significant inhibition of Raf-1 could only be accomplished by combined obstruction of two or more of these routes. Research into these matters is ongoing and will likely bear some refreshingly novel insights into what has proven to be rather cryptic signal transduction. Ultimately, we will have to move away from the imperfect specificity of the enzyme inhibitors and progress to more reliable and selective tools, such as dominant negative forms and antisense oligonucleotide-mediated knockdown of these enzymes.

Eventually, the entire molecular mechanism of adenylyl cyclase superactivation in all the relevant human tissues will be mapped and documented. Our goal is to understand this ubiquitous and vital compensatory response so that we can ultimately create drugs that activate G-protein-coupled receptors without the loss of responsiveness that occurs with current therapies. With this in mind, we have striven to significantly contribute our time and effort in hopes of one day alleviating pain and suffering.

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